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Direct Evidence That Genetic Variation in Glycerol-3-Phosphate and Malate Dehydrogenase Genes (*Gpdh* and *Mdh1*) Affects Adult Ethanol Tolerance in *Drosophila melanogaster*

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ABSTRACT

Many studies of alcohol adaptation in *Drosophila melanogaster* have focused on the *Adh* polymorphism, yet the metabolic elimination of alcohol should involve many enzymes and pathways. Here we evaluate the effects of glycerol-3-phosphate dehydrogenase (*Gpdh*) and cytosolic malate dehydrogenase (*Mdh1*) genotype activity on adult tolerance to ethanol. We have created a set of *P*-element-excision-derived *Gpdh*, *Mdh1*, and *Adh* alleles that generate a range of activity phenotypes from full to zero activity. Comparisons of paired *Gpdh* genotypes possessing 10 and 60% normal activity and 66 and 100% normal activity show significant effects where higher activity increases tolerance. *Mdh1* null allele homozygotes show reductions in tolerance. We use *piggyBac* FLP–FRT site-specific recombination to create deletions and duplications of *Gpdh*. Duplications show an increase of 50% in activity and an increase of adult tolerance to ethanol exposure. These studies show that the molecular polymorphism associated with GPDH activity could be maintained in natural populations by selection related to adaptation to alcohols. Finally, we examine the interactions between activity genotypes for *Gpdh*, *Mdh1*, and *Adh*. We find no significant interlocus interactions. Observations on *Mdh1* in both *Gpdh* and *Adh* backgrounds demonstrate significant increases in ethanol tolerance with partial reductions (50%) in cytosolic MDH activity. This observation strongly suggests the operation of pyruvate–malate and, in particular, pyruvate–citrate cycling in adaptation to alcohol exposure. We propose that an understanding of the evolution of tolerance to alcohols will require a system-level approach, rather than a focus on single enzymes.

THE genus Drosophila has an evolutionary history of exposure to alcohols, and it is believed that the adaptation to alcohols has facilitated the cosmopolitan spread of *Drosophila melanogaster* to temperate environments (Geer *et al.* 1993). Both larval and adult fruit flies feed on yeast, and this ecological niche exposes them to toxic fermentation products, including alcohols. In particular, it is believed that the high tolerance of *D. melanogaster* to alcohols is an evolved phenotype because other members of the *melanogaster* subgroup, such as *D. simulans*, show lower tolerance and avoid alcohol exposure (McKenzie and Parsons 1972; David and Bocquet 1975). In contrast, *D. melanogaster* utilizes ethanol as a carbon source and adult tolerance is highest in temperate climates (Cohan and Graf 1985), suggesting either increasing exposure to, or increased utilization of, alcohols in these regions. As a complex quantitative phenotype, both larval and adult alcohol tolerances show significant genetic variance (Cohan and Graf 1985; Cohan and Hoffmann 1986). Over several decades, this example of adaptation to a novel niche, one constituting both a resource and an environmental stress, has become a paradigm in evolutionary genetics.

The power of joining genetics and molecular analysis has made Drosophila an established model in studies of alcohol metabolism and tolerance. The induction of behaviors that are similar to those in humans is well noted, as are the parallels with alcohol metabolism in mammals (Scholz *et al.* 2000). In particular, there are two facets of alcohol tolerance that have been studied using Drosophila as a model. The first addressed short-term acquisition of tolerance, measured as a shift in knockdown time following a period of ethanol exposure (Scholz *et al.* 2000, 2005). The second (the focus of this study) is the metabolic elimination of alcohol and its relationship to tolerance and survival (Geer *et al.* 1993). Most of this second focus has centered on the relationship of biochemical variation in the alcohol dehydrogenase

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gene (*Adh*) to tolerance in both adults and larvae (Geer *et al.* 1993). Such studies have led to the textbook story of the *Adh* allozyme polymorphism (Freeman and Herron 2004; Futuyma 2005). However, the study of ADH has followed a path set down more by historical precedence than by design. ADH was the first enzyme system in Drosophila in which histochemical staining was used to detect electrophoretic variants (Johnson and Denniston 1964), and *Adh* was one of the first Drosophila genes cloned in the late 1970s (Kreitman 1983). Unfortunately, this precedence of *Adh* has directed interest away from the study of the development of metabolic tolerance to ethanol as a larger-scale problem involving many genes and pathways. The rapid elimination of ingested alcohols and its metabolic products is a system-wide challenge and must involve downstream pathways and metabolic networks, with possible interactions—all kept in redox balance.

In Drosophila, other genes and pathways have been implicated in ethanol tolerance (Van der Zel *et al.* 1991; Pecsenye and Saura 1998; Montooth *et al.* 2006; Morozova *et al.* 2006, 2007). For example, it was shown the next enzyme downstream, aldehyde dehydrogenase (*Aldh*), also plays a role in the subsequent metabolism of acetaldehyde to acetate in *D. melanogaster* larvae (Fry and Saweikis 2006; Fry *et al.* 2008). Glycerol-3-phosphate dehydrogenase (*Gpdh*) is another gene implicated in ethanol tolerance (Geer *et al.* 1993); a common allozyme polymorphism is found in natural populations. The derived $Gpdh^S$ allele possesses increased GPDH activity and is more common in temperate latitudes (Oakeshott *et al.* 1982, 1984; Sezgin *et al.* 2004). Furthermore, ADH and GPDH activity levels are coordinately induced in larvae exposed to alcohols (Geer *et al.* 1983; Lissemore *et al.* 1990). In population cage experiments, allozyme polymorphisms for both genes, as well as cytosolic malate dehydrogenase (*Mdh1*), responded to ethanol exposure over time (Cavener and Clegg 1978). These observations all imply that these other enzymes may play roles in adaptation to alcohols.

The hypothesis that *Gpdh* and *Mdh1* are involved in ethanol tolerance has not been directly tested using partial or full knockout alleles in rigidly controlled genetic backgrounds. To test this hypothesis, we use sets of *P*-element-excision alleles of the *Gpdh* (Merritt *et al.* 2006) and *Mdh1* genes to determine if reductions in GPDH and cytosolic MDH activity influence adult tolerance to alcohol. Furthermore, since in natural populations the higher-activity $Gpdh^S$ allele geographically covaries with the higher activity Adh^F allele, we also examine the effect of increases in GPDH activity by creating *Gpdh* gene duplications using *piggyBac* transposon insertions and the FLP–FRT site-specific recombination system (Parks *et al.* 2004). Finally, we explore the possibility of gene interactions among *Gpdh*, *Adh*, and *Mdh1* and their effect on ethanol tolerance.

MATERIALS AND METHODS

Lines: The *Gpdh* lines are described in Merritt *et al.* (2006). They consist of three alleles derived from mobilization of the *KG02555 P*-element insertion: $Gpdh^{\Delta 9.2}$, $Gpdh^{\Delta 24.1}$, and $Gpdh^{\Delta 10.2}$, with 0, 21, and 100% activities relative to normal. The progenitor allele in this line is the $Gpdh^F$ allele. Their *white*-marked *X* chromosomes are derived from Bloomington stock 2475, *w*;T(2;3)ap^{Xa}/Cy;TM3, Sb^1*, and the third chromosome backgrounds are replaced by using marker-assisted introgression in inbred line *w;CyO/Tft;VT83*.

The *Mdh1* alleles are created using excision of the *EY08761 P*-element insertion in gene CG5362. This insertion site lies inside the 5′-UTR, 12 bases upstream of the start codon. $Mdh1^{\Delta 18.1}$ has lost the mini-white construct, but retains >5 kb of the *P* element. In wild-type flies, ~15% of the crude MDH activity is cytosolic, while the remainder represents mitochondrial MDH2 leakage during homogenization (Hay and Armstrong 1976). The loss of cytosolic MDH enzyme activity in $Mdh1^{\Delta 18.1}$ is clearly seen after electrophoresis and allozyme staining (data not shown). $Mdh1^{\Delta 10.5}$ is a precise excision and recovers full gene activity. The *X* chromosome is the *white*-marker chromosome from Bloomington stock 2475, and the third chromosome is from *VT46*.

The *Adh* test alleles are derived from mobilization of the *KG05345 P* element that is inserted in exon 3. $Adh^{\Delta 25}$ is a partial excision that retains a small piece of the *P* element in exon 3 and possesses no ADH activity. $Adh^{\Delta 17}$ is a precise excision and possesses activity equal to a normal *Fast Adh* allele. The *X* chromosome is from Bloomington line 2475, and the third chromosome is replaced by that from inbred line *w;CyO/Tft;VT83*.

Lines *VT46* and *VT83* are derived from inbred lines collected in 1997 in Whiting, Vermont. Line *w;6326;6326.1* is a derivative of Bloomington stock 6326 that has the *X* chromosome from Bloomington stock 2475.

P elements were excised in male flies using standard dysgenic crosses (Merritt *et al.* 2006). Excision chromosomes (indicated by flies with white eyes) were isolated using the balancer chromosome *CyO*. Approximately 80–100 excision lines were sampled for each dysgenic cross. Relative allele function was determined by direct spectrophotometric assay of crude mass-adjusted enzyme activity (see below). Interline crosses were used to create heterozygotes and to test additivity in allele combinations in the event that transvection effects were present (Merritt *et al.* 2005). PCR and sequencing with flanking primers were used to determine molecular changes in the gene. All full-activity alleles were confirmed to have sequences consistent with the "precise" excision or gene conversion to a normal sequence. Reduced activity alleles possessed a spectrum of molecular changes from deletion of entire exons to retention of large pieces of the original *P* element. None of the alleles show single residue changes in amino acid sequence and thus catalytic function. Paired test genotypes differ only in the gene of interest.

A deletion-duplication series of *Gpdh* alleles was created using FRT–FLP-driven recombination (Parks *et al.* 2004) between *piggyBac* transposon insertions *f00109* and *e03988*. These insertions are ~40 kb apart and, upon FLPase-induced FRT recombination, will delete eight genes or duplicate seven genes. None of these other genes has an obvious relationship to ethanol tolerance. Eighty potentially recombinant lines were collected and screened by eye color, viability, PCR products, and GPDH activity. Chromosomes were genetically extracted using the *CyO* balancer chromosome and a subset of 40 chromosomes, which included 6 lethals, were further screened using PCR primer combinations and sequencing designed to detect hybrid *piggyBac* elements resulting from

recombination between FRT sites. Five lethals were deletions. Three lines were duplications, including one lethal. The recovery rate for both deletions and duplications was ~10%. All second chromosomes had the *X* and third chromosome backgrounds replaced using line *w*; 6326.*6326.1*. The progenitor allele in this line is $Gpdh^S$ and is already in the *6326* second chromosome.

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 five individuals pooled in 1 ml of grinding 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 assays. 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, each of 10 replicate samples were assayed twice and the average was used as an estimate of each genotype activity. Enzyme activity is expressed as nanomolars of NAD^+ reduced/min/fly (see Merritt *et al.* 2006). 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, 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.

Crosses to set up test genotypes: All flies were reared on standard cornmeal media in 200-ml plastic flasks. In two sets of experiments, test genotypes were created with alleles $Gpdh^{\Delta 9.2}$, $Gpdh^{\Delta 24.1}$, and $Gpdh^{\Delta 10.2}$. In experiment 1, $Gpdh^{\Delta 9.2}$ and $Gpdh^{\Delta 10.2}$ males were mated with $Gpdh^{\Delta 24.1}$ females producing genotypes with 15 and 60% activities relative to a *10.2/10.2* genotype. The *10.2/10.2* genotype possesses activity that is ~12% higher than the average GPDH activity of the 10 wild second chromosome lines assayed in Merritt *et al.* (2006). In experiment 2, $Gpdh^{\Delta 9.2}$ and $Gpdh^{\Delta 10.2}$ males (50 each) were separately mated with *w; 6326;6326.1* females (100 each), producing genotypes with 66 and 100% relative GPDH activities. The *6326/6326* genotype possesses GPDH activity that is 25% higher than *10.2/10.2* and possesses the $Gpdh^S$ allele. Densities were standardized in each bottle. Emerging males were collected from multiple replicate bottles, pooled by genotype, aged 4–6 days, and used in the assay. $Gpdh^{\Delta 9.2}$ homozygous genotypes were not tested because the homozygous null GPDH genotypes possess very low viability (Merritt *et al.* 2006).

For *Mdh1* testcrosses in experiment 3, alleles *18.1* and *10.5* were combined to create 0, 50, and 100% normal MDH activity genotypes using the same rearing and collection methods as for *Gpdh*. All *Mdh1* genotypes bear the EY *P*-element progenitor second chromosome and the *white*-marked *X* and *VT46* third chromosomes.

For *Adh* testcrosses in experiment 4, the $Adh^{\Delta 17}$ and $Adh^{\Delta 25}$ alleles were combined to create 0, 50, and 100% normal ADH activity genotypes. All *Adh* genotypes bear the KG progenitor second chromosome and the *white*-marked *X* and *VT83* third chromosomes.

In the *Gpdh* duplication series crosses for experiment 5, the $Gpdh^{pB10}$ and $Gpdh^{pB23}$ alleles were used as representative single-copy and duplicate alleles and combined to produce three genotypes: $Gpdh^{pB10}/Gpdh^{pB10}$, $Gpdh^{pB10}/Gpdh^{pB23}$, and $Gpdh^{pB23}/Gpdh^{pB23}$ with 100, 125, and 150% relative GPDH activities. These lines all bear the same *white*-marked *X* chromosome (as the previous lines) and the *6326.1* second and third chromosomes.

In experiment 6, $Mdh1^{18.1}$ and $Mdh1^{10.5}$ males (50 each) were separately mated with $Adh^{\Delta 17}$ and $Adh^{\Delta 25}$ homozygous females to create four MDH:ADH genotypes with predicted 50:50, 50:100, 100:50, and 100:100 normal activity genotypes. In experiment 7, $Mdh1^{18.1}$ and $Mdh1^{10.5}$ homozygous females (50 each) were separately mated with $CyO/Gpdh^{\Delta 9.2}$ and $Gpdh^{\Delta 10.2}$ males to create four MDH:GPDH genotypes with predicted 50:50, 50:100, 100:50, and 100:100 normal activity genotypes. In experiment 8, $CyO/Gpdh^{\Delta 9.2}$ and $Gpdh^{\Delta 10.2}$ males (50 each) were separately mated with $Adh^{\Delta 17}$ and $Adh^{\Delta 25}$ homozygous females to create four GPDH:ADH activity genotypes with 50:50, 50:100, 100:50, and 100:100 normal activity genotypes. Enzyme assays of emerging flies indicate that these activity ratios are present as expected.

Basic tolerance assay: The ethanol tolerance assay included replicated vials each with 10 adult males aged 5–7 days. A standard-sized cotton ball was pressed into the bottom of each vial and saturated with 2.5 ml of a solution of 2% sucrose and 15% ethanol. Vials were checked at two 24-hr intervals, recording the number of dead flies appearing over the 2-day interval. If <10% average mortality was observed, counts were extended another day. No significant mortality was ever observed for control flies (maintained on 2% sucrose over a 3-day period).

Statistics: Statistical analysis was carried out on arc–sine transformed measures of the percentage surviving. Single-nested ANOVAS (single-locus tests), two-way ANOVAs (for di-locus interactions), 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).

RESULTS

Experiments 1 and 2 compare the effects of GPDH activity reduction on ethanol tolerance. In each test comparison, there is a paired reference genotype with GPDH activity that scales within the normal range (Merritt *et al.* 2006). The first experiment (Figure 1A, shaded bars) contrasted *Gpdh* genotypes with 15% (*9.2/24.1*) and 60% (*10.2/24.1*) activity relative to a *10.2/10.2* genotype. Clearly, what is "normal" activity is arbitrary here because GPDH activity varies across wild alleles and backgrounds. The *10.2/10.2* genotype has 12% higher activity than the average for the 10 wild second chromosome lines reported in Merritt *et al.* (2006). Over the 48 hr of ethanol exposure, the survival rate of the low-activity genotype is less than one-half of the high-activity genotype ($F_{1,36} = 11.83$, $P < 0.0015$). Experiment 2 (Figure 1A, solid bars) compared genotypes constructed by crossing *10.2* and *9.2* males with *6326* females. The *10.2/6326* reference genotype possesses GPDH activity again in the normal range (~12% higher than *10.2/10.2*) and the *6326* line possesses the $Gpdh^S$ allele. The *9.2/6326* genotype has a relative activity that is 66% of *10.2/6326* (Figure 1A) There is a highly significant difference in ethanol tolerance ($F_{1,31} = 7.78$, $P < 0.009$). These two experiments show a reduction in tolerance with lower GPDH activity.

Combining the $Mdh1^{\Delta 18.1}$ and $Mdh1^{\Delta 10.5}$ alleles, we created genotypes with 0, 50%, and full cytosolic MDH activities in experiment 3. There was a highly significant effect of *Mdh1* genotype on ethanol tolerance (Figure 1B; $F_{2,105} = 14.69$, $P < 0.0001$). This was attributed to the

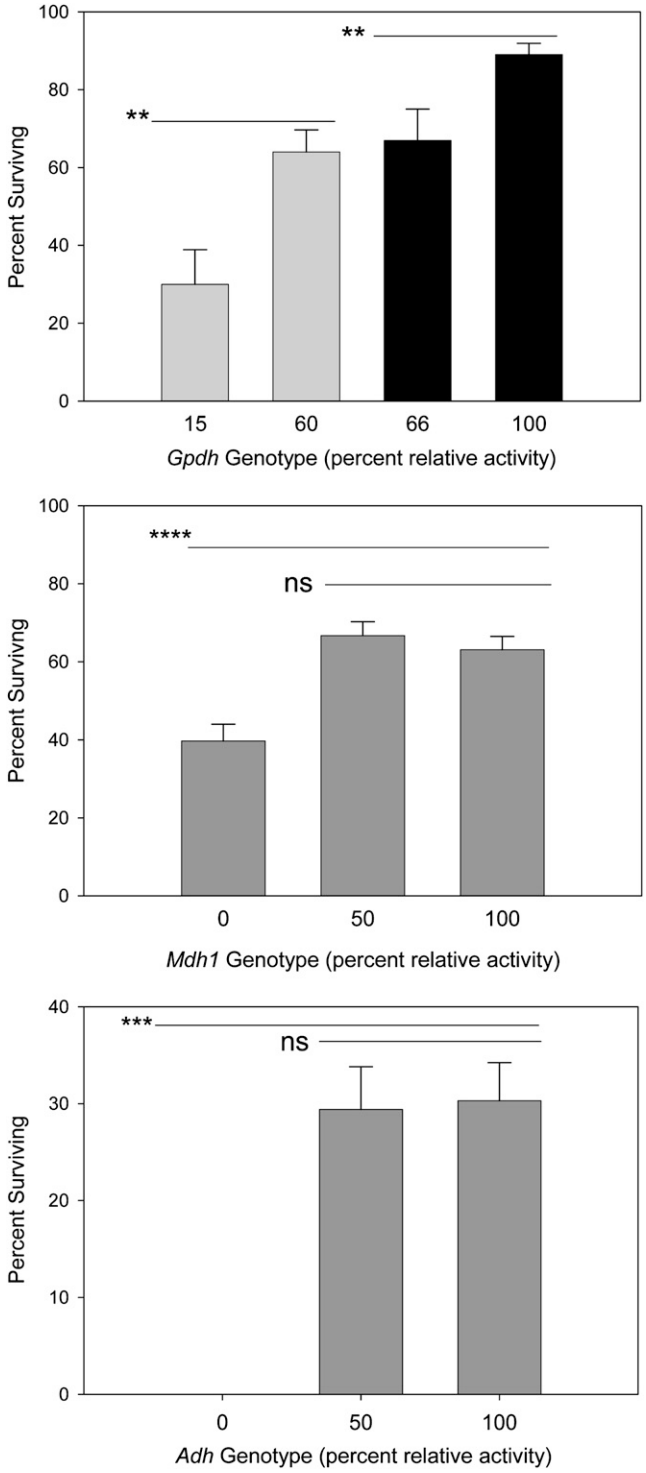

Figure 1.—Genotype-specific adult (male) survivorship after 48 hr of exposure to a 15% ethanol, 2% sucrose solution. (A) Experiments 1 and 2 using genotypes that possess 10 and 60% normal GPDH activity (shaded bars)—*Gpdh9.2/24.1* ($n = 17$) and *Gpdh24.1/10.2* ($n = 20$)—and (solid) genotypes *Gpdh 9.2/6326* ($n = 13$) and *10.2/6326* ($n = 20$) possessing 66 and 100% normal activity. (B) Experiment 3 using *Mdh1* genotypes *18.1/18.1* ($n = 33$), *18.1/10.5* ($n = 39$), and *10.5/10.5* ($n = 36$), representing 0, 50, and 100% normal MDH activities. The homozygous null genotype has a significantly lower survival rate ($P < 0.0001$). (C) Experiment 4 using three *Adh* genotypes: *25/25* ($n = 29$), *17/25* ($n = 33$), and *17/17* ($n = 35$) possessing 0, 50, and 100% normal ADH activity. Error bars represent ±1 SE. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

full homozygous null *Mdh1* genotype, *18.1/18.1*, which possessed significantly reduced tolerance relative to the 50 and 100% activity genotypes.

Experiment 4 using the $Adh^{\Delta 25}$ (null) and $Adh^{\Delta 17}$ alleles found a highly significant effect with the homozygous null genotypes showing significantly lower tolerance (Figure 1C; $F = 21.35$, $P < 0.001$). However, there was no significant difference in tolerance between the 50 and 100% activity genotypes after 48 hr.

In experiment 5, using *piggyBac* FRT–FLP-facilitated recombination, we duplicated an 8-kb region spanning the *Gpdh* gene and placed these alleles in isogenic *X* and third chromosome backgrounds. The progenitor chromosomes for the *piggyBac* insertions are the *6326* line. The GPDH activities of the final duplication-deletion *Gpdh* allele sets are shown in Figure 2A. The duplicated alleles, *pB16* and *pB23*, possess a 50% activity increase over single-copy alleles. When tested for ethanol tolerance (Figure 2B) using alleles *pB10* and *pB23*, we observed a highly significant effect of *elevated* GPDH activity ($F_{2,83} = 7.45$, $P < 0.001$).

Experiments 6, 7, and 8 address interactions in dilocus combinations that yield full and half-full activity genotypes (Figure 3, A–C). With respect to tolerance, there were no significant interactions between genotypes in any experiment. Interestingly, there are highly significant main effects of the *Mdh1* genotype ($Mdh1^{18.1}$ and $Mdh1^{10.5}$) in both *Adh* (Figure 3A; $F_{1,73} = 21.9$, $P < 0.0001$) and *Gpdh* (Figure 3B; $F_{1,10} = 8.0$, $P < 0.038$) backgrounds. The lower-activity *Mdh1* genotype has significantly higher tolerance. This is suggested in experiment 3 as well (see Figure 1B). *Adh* genotypes showed significant genotype effects with the higher-activity $Adh^{\Delta 17}$ allele possessing increased tolerance in combination with both *Mdh1* genotypes (Figure 3A; $F_{1,73} = 6.00$, $P < 0.017$), but was not significant in combination with the *Gpdh* genotypes (Figure 3C). $Gpdh^{\Delta 9.2}$ and $Gpdh^{\Delta 10.2}$ genotypes were not significant in either background (Figure 3C), although the differences in the *Adh* background ($F_{1,110} = 2.71$, $P < 0.102$) are consistent with higher ethanol tolerance associated with the high-activity *Gpdh* genotype in both tests.

In summary, in the five experiments that assessed *Gpdh* genotype effects all showed *increased* tolerance with *increasing* activity and three were statistically significant. In the three experiments in which the *Mdh1* genotype effects were tested, all showed *increasing* tolerance with a 50% *reduction* in cytosolic MDH activity and two were statistically significant. While there are no statistically significant interactions in the strictest sense, these studies raise the possibility of the impact of genetic background on tolerance.

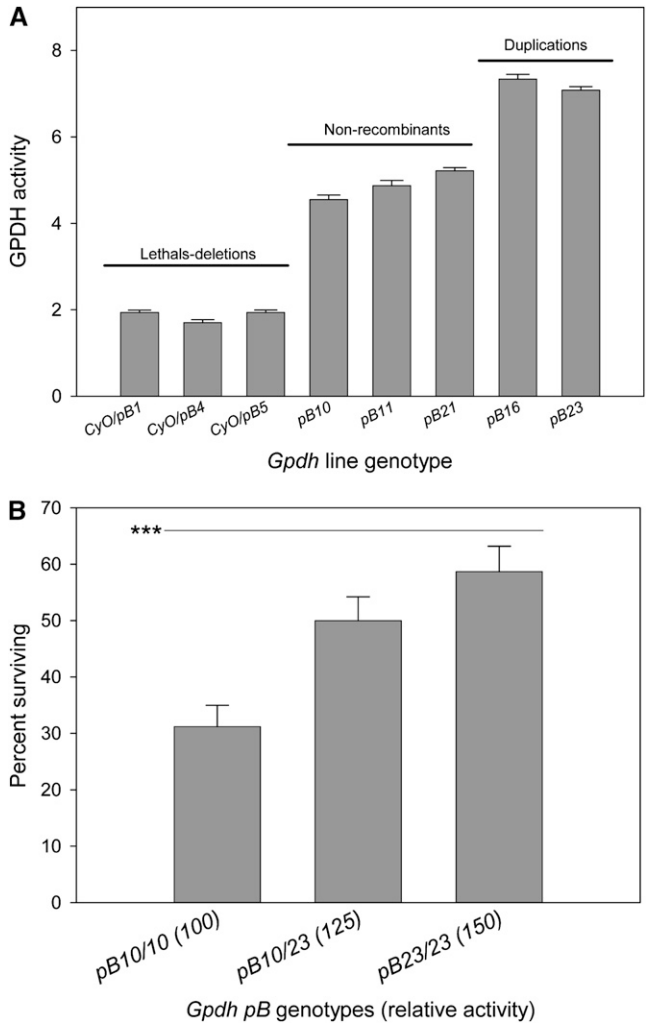

FIGURE 2.—*Gpdh* gene duplication and deletion by *piggyBac* FLP–FRT site-specific recombination and effects on ethanol tolerance. (A) The GPDH activities as ΔOD units for seven *piggyBac* (*pB*) alleles recovered in the FRT–FLP recombination crosses and confirmed by diagnostic PCR and direct sequencing. All deletions are semilethal and balanced over *CyO*. (B) Experiment 5 showing the percentage of survival of three *Gpdh* genotypes possessing 100% ($n = 34$), 125% ($n = 28$), and 150% ($n = 24$) relative GPDH activity to normal. Among genotypes, differences are statistically significant by ANOVA on arc–sine transformed values ($F_{2,83} = 7.44$, $^{***}P < 0.001$). Error bars represent ±1 SE.

DISCUSSION

When adult flies possess no alcohol dehydrogenase activity, exposure to ethanol vapors results in knockdown within minutes, as often reported and again confirmed in our experiment. This sensitivity to ethanol exposure emphasizes the need for rapid elimination of ethanol, which requires not only the functioning of the initial ADH and ALDH steps and the downstream elimination of products, but also cofactor regeneration. Because both initial steps in ethanol breakdown consume NAD and produce NADH (two moles of NADH for each mole of ethanol), an essential consideration in ethanol metabolism is the maintenance of the redox potential in the cell. In Drosophila larvae exposed to dietary ethanol, there is a notable shift in the NADH: NAD ratio (Geer *et al.* 1983) and in adults a drop in NAD levels during 24 hr of exposure (McElfresh and McDonald 1983). In mammals, the maintenance of cellular redox balance is a central challenge in mammalian alcohol detoxification as well (Berry *et al.* 1994), and an important mechanism for the restoration of the redox balance in mammals is the malate–aspartate shuttle. In insects, where the glycerol phosphate shuttle is believed to have a major role in transferring NADH equivalents into the mitochondria, GPDH should be important in an analogous fashion.

In a series of early cage experiments on allozyme polymorphisms for *Adh*, *Gpdh*, and *Mdh*, Cavener and Clegg (1978) replicated selection under ethanol exposure in supplemented food in experiments that ran >50 generations. Both *Adh* and *Gpdh* showed repeatable responses, indicating selection favoring the Adh^F and $Gpdh^S$ allozyme alleles in the ethanol-exposed cage populations. Control populations showed no effective response, and cage populations removed from ethanol selection (relaxed) ceased allele changes. *Mdh1* allozyme frequencies did not immediately respond to alcohol exposure and were followed less closely, but at generation 57 both ethanol-exposed populations were fixed for the Mdh^S allele, while controls were still polymorphic. This allele is most common in natural populations, typically <97.5% (Hay and Armstrong 1976). These studies suggest a participation of not just *Adh*, but *Gpdh* and possibly *Mdh1* in the adaptation to alcohols in natural populations. However, as typical of cage experiments and as noted by the investigators, lines were started with small samples of wild chromosomes, and the initial linkage disequilibrium associated with this sampling potentially confounds interpretation. These demographic effects can be avoided by direct manipulation of enzyme levels as we have done here.

Our results show that after >48 hr of ethanol exposure adult male ethanol tolerance can depend on the activity levels of GPDH. We have also shown that changes in tolerance can be affected by both decreases and increases in GPDH activity relative to expected "normal" levels. Demonstrating this latter observation is important because in natural populations the derived $Gpdh^S$ electrophoretic allele consistently shows 20% higher activity than the $Gpdh^F$ allele (Miller *et al.* 1975; Laurie-Ahlberg and Bewley 1983; Bewley *et al.* 1984; Kang *et al.* 1998). Furthermore, the $Gpdh^S$ allele frequency increases with latitude (Miller *et al.* 1975; Oakeshott *et al.* 1982; Sezgin *et al.* 2004), consistent with the hypothesis that increased activity is associated with increased tolerance in temperate climates. The intrapopulation sequence variation for *Gpdh* shows features associated with historical balancing selection and high levels of silent polymorphism relative

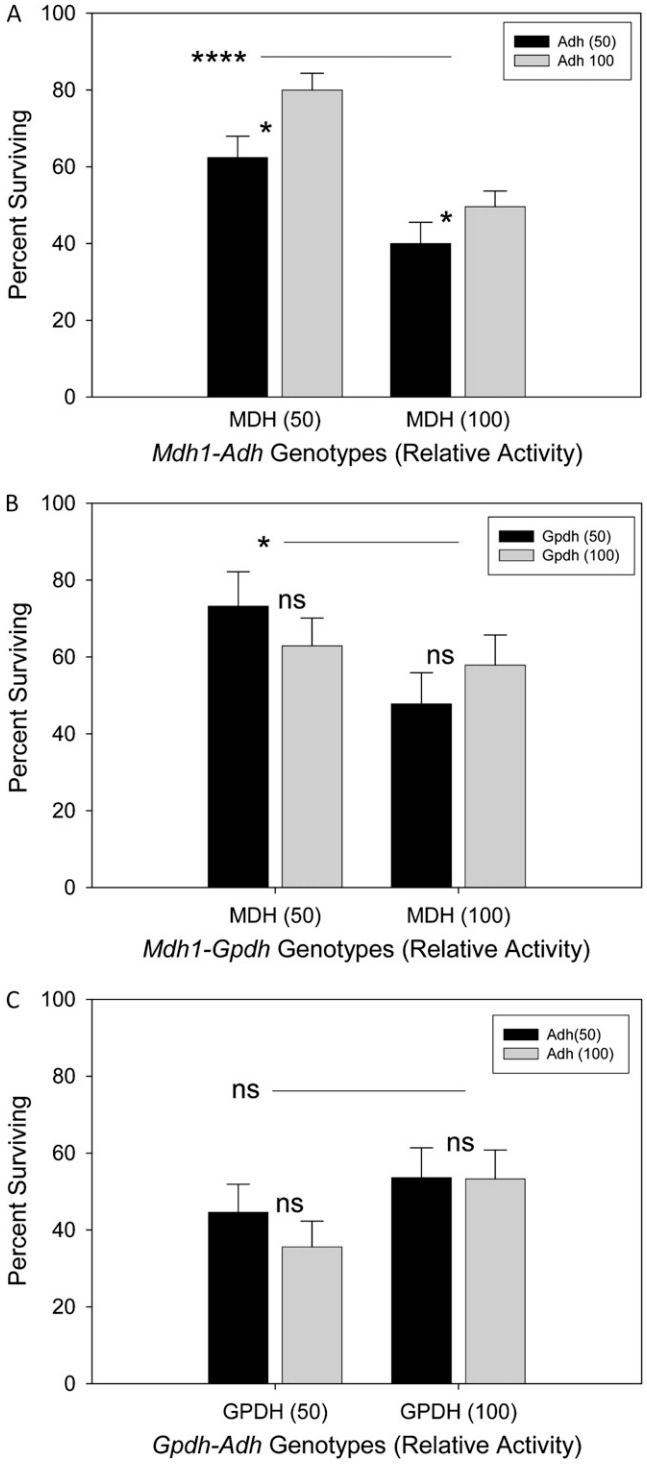

FIGURE 3.—The ethanol tolerance as percentage of survival of *Mdh1*, *Adh*, and *Gpdh* di-locus genotypes. No experiments found statistically significant interactions. (A) Experiment 6 using *Mdh1* and *Adh* genotypes with ratios of 50:50 (n = 15), 50:100 (n = 23), 100:50 (n = 16), and 100:100 (n = 26) normal activities. There are significant *Mdh* genotype effects (****$P < 0.0001$) and significant *Adh* genotype effects (*$P < 0.017$). (B) Experiment 7 using *Mdh1* and *Gpdh* genotypes with ratios of 50:50 (n = 22), 50:100 (n = 34), 100:50 (n = 27), and 100:100 (n = 29) normal activities. There are significant *Mdh* genotype effects (*$P < 0.038$). (C) Experiment 8 using the *Gpdh* and *Adh* genotypes with ratios of 50:50 (n = 28), 50:100 (n = 34), 100:50 (n = 25), and 100:100 (n = 27) normal activities. Neither *Gpdh* nor *Adh* genotype effects were significant. The expected relative activities of genotypes are shown inside parentheses. Error bars represent ±1 SE.

to the associated interspecific divergence (TAKANO *et al.* 1993; KREITMAN and AKASHI 1995), similar to the situation at *Adh* (HUDSON *et al.* 1987, but see BEGUN *et al.* 1999).

Because the relationship between metabolic flux and enzyme activity is often expected to be hyperbolic (HARTL *et al.* 1985; DYKHUIZEN *et al.* 1987), it does not necessarily follow that increases in activity above normal would also show enhanced tolerance. To test this hypothesis, we have used the *piggyBac* transposon and FLP–FRT recombination to produce a duplication of the *Gpdh* region and show that a 50% increase in GPDH activity (as seen for the derived $Gpdh^s$ allele) causes increased tolerance to ethanol. While this method was introduced by PARKS *et al.* (2004) to create site-specific deletions, our study is the first reported use of this genetic tool to increase gene function through duplication.

It is unclear if increased tolerance associated with *Gpdh* activity results from better maintaining the redox balance, increased triglyceride accumulation, or both. In a microarray study of genes induced under exposure to ethanol in adults (MOROZOVA *et al.* 2006), both *Gpdh* and its mitochondrial shuttle partner, *Gpo-1*, show strong induction of transcripts. In larvae, GPDH is also strongly induced under dietary ethanol along with the accumulation of triglycerides (GEER *et al.* 1983; LISSEMORE *et al.* 1990). However, while the wild-type larval NADH:NAD ratio increases under ethanol exposure (0.22–0.36), this shift was not significantly different in *Gpdh* null genotypes (GEER *et al.* 1983). On sucrose control diets, both null *Gpdh* and wild-type larvae possess equal cofactor concentrations. However, under ethanol exposure, wild-type larvae see a 22% increase in total cofactor concentrations, but *Gpdh* null larvae experience a 24% drop. Therefore, on ethanol diets *Gpdh* null genotypes possess only 61% of the combined cofactor concentration of wild-type larvae. If the same phenomenon exists in adults, then the gain of tolerance with increased GPDH activity may derive from increased concentrations of both NADH and NAD, and not from the redox balance.

The different genotype-specific effects across experiments suggest a dependency on genetic background. Within each experiment, line constructions vary only in the targeted genes, but *between* experiments genotypes have different genetic backgrounds (the unique progenitor chromosomes of the KG, EY, and *piggyBac* elements), and the different outcomes certainly raise the possibility of genomewide interactions. In experiments 1–3, the higher-activity *Gpdh* genotypes always possess greater tolerance. In the

interaction experiments, where *Mdh1* and *Adh* genotypic backgrounds are varied, the *Gpdh* main effects are again in the same direction but nonsignificant. They certainly suggest further potential interactions. There are likely to be numerous other genes capable of participating in ethanol tolerance (Morozova *et al.* 2006, 2007), and genetic variation in these could contribute to background effects and differences between experiments.

The role of variation in cytosolic MDH activity in conferring ethanol tolerance appears complex. Complete loss of activity results in reduced ethanol tolerance (homozygous null *Mdh1* genotypes are still normal in viability and fecundity), but genotypes with half-normal MDH activity clearly show significant increases in tolerance. Therefore, partial reduction of cytosolic MDH must enhance the elimination or metabolism of ethanol. In mammals, the malate–aspartate shuttle is responsible for the transfer of NADH equivalents into the mitochondria and the maintenance of redox balance, but its action in Drosophila is unknown. The cytosolic and mitochondrial glutamate–oxalacetate transaminases necessary for the shuttle are abundant, as is the aspartate–glutamate carrier (*Aralar1*). If this shuttle is present in flies, then a reduction in tolerance due to the complete loss of cytosolic MDH activity is understandable. However, the increased tolerance with partial reductions in activity is not expected and requires a different explanation. One alternative hypothesis is that *Mdh1* plays a less direct role and the associated shuttle is an energy-state signal in Drosophila, triggering top–down responses as it clearly does in insulin secretion in the pancreatic β-cells (Rubi *et al.* 2004).

There is good evidence that potential malate–pyruvate and pyruvate–citrate cycles (Farfari *et al.* 2000; Guay *et al.* 2007) are strongly induced after adult response to ethanol. This is because Morozova *et al.* (2006) noted strong increases (nearly twofold) in transcription response for cytosolic malic enzyme (*Men*), but especially phosphoenopyruvate carboxykinase (*Pepck*) and the pyruvate carboxylase gene (CG1516). These cycles would act in the metabolic elimination of ethanol-derived acetyl-CoA as mitochondrial effluxes of malate and citrate. The cytosolic citrate is converted into oxalacetate and malonyl-CoA by ATP-citrate lyase, and oxalacetate is returned by PEPCK to gluconeogenesis and used in triglyceride formation. Malonyl-CoA is directed toward lipid synthesis (Freriksen *et al.* 1994). However, the efflux of citrate by the tricarboxylate carrier requires an exchange of malate; therefore, it is apparent that reduction of the pyruvate–malate shuttle could increase tolerance if the major detoxification pathway uses lipid and triglyceride synthesis. Since MDH1 metabolically bridges PEPCK and MEN, activity variation in it could control their relative roles in ethanol metabolism.

The association of increased alcohol tolerance with low cytosolic MDH activity certainly reflects a potential for natural selection to act on *Mdh1* activity levels in natural populations, but, unlike *Gpdh* and *Adh*, there is no DNA sequence-based evidence that the *Mdh1* gene is responding to positive or balancing selection in *D. melanogaster*. There is no common amino acid polymorphism and no evidence for clines in SNP sites inside the *Mdh1* gene (Sezgin *et al.* 2004). This does not rule out regulatory variation polymorphism affecting enzyme levels, but this remains to be investigated. All population genetic evidence points to simple purifying selection. It is possible that general negative pleiotropic fitness effects associated with reduced MDH activity prevent its participation in naturally occurring variation in ethanol tolerance.

In natural populations of Drosophila, alcohol tolerance is a complex genetic trait and genetic variation for *Adh* has been shown to only partly contribute to the final phenotype (Cohan and Graf 1985). Clearly, genetic variation in many other enzymes should be important in the metabolic elimination of alcohols. *Adh*, *Aldh*, *Gpdh*, and *Mdh1* possess different levels and patterns of intraspecific polymorphism and interspecific divergence, and this emphasizes the distinction between (1) identifying pathways of potential detoxification and (2) finding points of genetic variation realized as an adaptive response. The former distinction depends on the individual idiosyncratic properties of the enzymes as well as their context in a system of pathways. However, the impact of natural selection acting on genes in adaptive response to alcohol-based fitness reduction will also depend on the pleiotropic effects on other fitness components and their trade-offs in nonalcohol environments. Nevertheless, a complete understanding of the evolution of tolerance to alcohols will require a large-scale or system-level approach, rather than a focus on single enzymes.

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