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Dietary Ethanol Mediates Selection on Aldehyde Dehydrogenase Activity in *Drosophila melanogaster*[1]

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SYNOPSIS. Ethanol is an important environmental variable for fruit-breeding *Drosophila* species, serving as a resource at low levels and a toxin at high levels. The first step of ethanol metabolism, the conversion of ethanol to acetaldehyde, is catalyzed primarily by the enzyme alcohol dehydrogenase (ADH). The second step, the oxidation of acetaldehyde to acetate, has been a source of controversy, with some authors arguing that it is carried out primarily by ADH itself, rather than a separate aldehyde dehydrogenase (ALDH) as in mammals. We review recent evidence that ALDH plays an important role in ethanol metabolism in *Drosophila*. In support of this view, we report that *D. melanogaster* populations maintained on ethanol-supplemented media evolved higher activity of ALDH, as well as of ADH. We have also tentatively identified the structural gene responsible for the majority of ALDH activity in *D. melanogaster*. We hypothesize that variation in ALDH activity may make an important contribution to the observed wide variation in ethanol tolerance within and among *Drosophila* species.

INTRODUCTION

Drosophila species which breed in fermenting fruit can encounter ethanol concentrations of up to 4–5% (Gibson *et al.*, 1981). Some *Drosophila* species breed in wineries and breweries, where ethanol concentrations may be even higher (McKenzie and McKechnie, 1979; Gibson *et al.*, 1981). Ethanol can serve as a resource at low concentrations, but at high concentrations is toxic (Parsons *et al.*, 1979). That ethanol has been an important selective agent for *Drosophila* is attested by the results of interspecific comparisons: species which normally breed in fruit are more resistant to the toxic effects of ethanol, and have higher activity of the enzyme alcohol dehydrogenase (ADH, E.C. 1.1.1.1), than species which breed in mushrooms and other non-sweet vegetation (Merçot *et al.*, 1994).

The well-studied species *D. melanogaster* appears to have evolved unusually high affinity for ethanol. Strains of *D. melanogaster* typically can tolerate higher concentrations of ethanol (David and Bocquet, 1975; Parsons *et al.*, 1979), and are more likely to be attracted to ethanol (McKenzie and Parsons, 1972; Parsons, 1977), than strains of its sibling species *D. simulans*, from which *D. melanogaster* diverged within the last 3 million years (Powell, 1997). The ethanol tolerance of *D. simulans* is typical of other *melanogaster* subgroup species (Merçot *et al.*, 1994). Response to ethanol is not uniform within *D. melanogaster*, however. Populations from high latitudes, whether in the Northern or Southern hemisphere, are more ethanol tolerant than tropical populations (David and Bocquet, 1975; Anderson, 1982). *D. simulans* populations show no such cline (David and Bocquet, 1975); thus the difference in ethanol tolerance between *melanogaster* and *simulans* is greater in temperate zones than in the tropics. On a smaller geographical scale, progeny of *D. melanogaster* collected in breweries or wineries often show higher ethanol tolerance than those of flies collected only a short distance away, giving evidence for local adaptation to high ethanol levels (reviewed in McKechnie and Geer, 1993).

Given the wealth of genetic tools available for working with *D. melanogaster*, the adaptation of this species to high ethanol levels on varying geographic scales provides an excellent system for studying the genetics of an ecophysiological adaptation. Previous work has focused extensively on the role of alcohol dehydrogenase, which catalyzes the reversible, NAD^+-dependent oxidation of ethanol to acetaldehyde (reviewed in Van Delden, 1982; Chambers, 1988; Geer *et al.*, 1993; Heinstra, 1993; Eanes, 1999). Two electromorphs, S and F, differing by one amino acid substitution, segregate in most *D. melanogaster* populations. The F allele confers greater ADH activity than the S allele, and in many studies has appeared to confer higher fitness in the presence of ethanol. The F allele also increases in frequency with latitude in both the northern and southern hemispheres. Nonetheless, variation in ADH activity in general, and the *Adh* polymorphism in particular, appear to explain only a small proportion of genetic variation in ethanol tolerance in *D. melanogaster*. For example, Anderson (1982) found that adult ethanol tolerance of isofemale lines collected from Australia was strongly correlated with latitude even after controlling for *Adh* allele frequency and ADH activity. In addition, in some cases, laboratory populations maintained on ethanol-supplemented food evolved increased ethanol tolerance without consistent changes in either ADH activity or *Adh* allele frequencies (Gibson *et al.*, 1979; Oakeshott *et al.*, 1983,

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1984). That such changes have been observed in some studies (*e.g.,* Gibson, 1970; Bijlsma-Meeles and Van Delden, 1974; Cavener and Clegg, 1978) but not others suggests that unknown genes may interact epistatically with *Adh* to determine fitness in the presence of ethanol.

Aldehyde dehydrogenase (ALDH, E.C. 1.2.1.3) is another enzyme that could contribute to variation in ethanol tolerance in *D. melanogaster*. ALDH catalyzes the irreversible, NAD^+-dependent oxidation of acetaldehyde to acetate (Weiner, 1979*b*). ALDH is believed to be the main enzyme responsible for the oxidation of acetaldehyde in mammals (Weiner, 1979*a*). In humans, an inherited deficiency in the mitochondrial ALDH isozyme, ALDH2, causes a syndrome known as acute alcohol sensitivity (Impraim *et al.*, 1982; Yoshida *et al.*, 1984; Peng *et al.*, 1999). Affected individuals, who are mostly of east Asian descent, experience a variety of unpleasant symptoms caused by the accumulation of acetaldehyde after ingesting small amounts of ethanol.

While it has been clear for some time that *Drosophila* contains an active ALDH (Liétaert *et al.*, 1985; Garcin *et al.*, 1986; Heinstra *et al.*, 1989; Anderson and Barnett, 1991; Leal and Barbancho, 1992), some workers have suggested that ALDH is less important for ethanol metabolism in *Drosophila* than in mammals (Geer *et al.*, 1985; Heinstra *et al.*, 1989). This claim followed the discovery that *Drosophila* ADH is capable of oxidizing acetaldehyde to acetate by itself (Heinstra *et al.*, 1983; Eisses *et al.*, 1985; Geer *et al.*, 1985; Moxon *et al.*, 1985; Heinstra *et al.*, 1989). In contrast, available evidence at the time indicated that mammalian ADHs, which are evolutionarily unrelated and structurally quite different from *Drosophila* ADH, possess at best a weak ability to oxidize aldehydes (Hinson and Neal, 1972). In apparent support of the view that ADH rather than ALDH is the main enzyme responsible for acetaldehyde oxidation, feeding an ALDH inhibitor to larvae resulted in a comparatively small reduction (12–19%) in the flux of ethanol into lipid (Heinstra *et al.*, 1989; Heinstra and Geer, 1991).

Other findings, however, have given evidence that *Drosophila* ALDH may be more important in ethanol metabolism than suggested by the above observations. First, studying adults, Leal and Barbancho (1992) found that inhibiting ALDH *in vivo* reduced survivorship in the presence of 5% ethanol from near 100% to 0%. While one interpretation of these results is that ALDH is more important in adults than it is in larvae (Geer *et al.*, 1993; Heinstra, 1993), similar toxicity tests in ALDH-inhibited larvae (as opposed to flux measurements using a low concentration of ethanol) do not appear to have been performed. Second, while ADH is a cytosolic enzyme, *Drosophila* ALDH has mitochondrial forms in both larvae (Garcin *et al.*, 1986) and adults (Liétaert *et al.*, 1985; Anderson and Barnett, 1991; Leal and Barbancho, 1993). Thus only ALDH is likely to be able to protect mitochondria from acetaldehyde poisoning. Finally, if ADH is the primary enzyme responsible for acetaldehyde oxidation *in vivo*, one would expect ADH-null mutants to be drastically more sensitive to acetaldehyde than wild-type flies. While ADH-null stocks show some reduction in LD50 for acetaldehyde, the reduction is comparatively small, and is likely to be at least partly due to the well-known ability of ADH to reduce acetaldehyde to the less toxic ethanol (Parsons and Spence, 1981; David *et al.*, 1984).

Another significant recent finding is that mammalian ADHs can carry out the oxidation of acetaldehyde to acetate at a rate much greater than previously suspected (Henehan and Oppenheimer, 1993; Svensson *et al.*, 1996). Moreover, the reaction carried out by both mammalian and *Drosophila* ADHs is not a simple oxidation of acetaldehyde to acetate, with corresponding reduction of NAD^+, but a dismutation reaction, whereby two molecules of acetaldehyde are converted to one each of ethanol and acetate in coupled half-reactions (Henehan *et al.*, 1995). At steady state under physiological pH, the dismutation results in no net reduction of NAD^+. Because the dismutation reaction continually regenerates ethanol from acetaldehyde, it seems unlikely that an organism would rely on it as the main strategy for acetaldehyde elimination, particularly when internal acetaldehyde concentrations are high.

Henehan *et al.* (1995) also showed that most previous attempts to measure the acetaldehyde-oxidizing ability of *Drosophila* ADH were compromised by a complex set of artifacts arising from the use of Tris or glycine buffers, which react with acetaldehyde and NAD^+ to produce spurious changes in absorbance, and high, non-physiological pH. As a result, reported kinetic parameters (Eisses *et al.*, 1985; Moxon *et al.*, 1985) for the acetaldehyde-oxidation reaction carried out by *Drosophila* ADH are not valid.

A previous study (Anderson and Barnett, 1991) gave evidence that ALDH activity varies considerably among wild-type *D. melanogaster* stocks, but nothing is known about whether such variation contributes to variation in ethanol tolerance. In addition, the structural locus or loci responsible for ALDH activity in *Drosophila* is not known. Here, we report a test of whether populations maintained on ethanol-supplemented media (Fry, 2001) evolved increased ALDH activity, as expected if ALDH is important in ethanol tolerance. In addition, we test whether a putative ALDH structural locus identified by the genome project is the source of most of the measurable ALDH activity in adult flies.

MATERIALS AND METHODS

Drosophila *populations and stocks*

We measured ALDH and ADH activity in eight populations descended from a single base population and maintained on a range of ethanol concentrations for many generations. The origin of the populations and general rearing conditions are described in Fry (2001). The two ''R'' populations have been main-

tained on regular *Drosophila* medium, while the two ''E'' populations have been maintained on medium supplemented with 12% ethanol. In the two ''M'' populations (for ''mixed''), half of the flies are reared on regular medium, and half on medium with 12% ethanol, each generation. After 95 generations, the populations ranked E > M > R and E ≅ M > R in egg-to-adult survival and development rate, respectively, on 12% ethanol (Fry, 2001). One-hundred generations after the R, E, and M populations were established, an ''HE'' (for ''high ethanol'') population was established from each of the two E populations; these are maintained on medium supplemented with 16% ethanol.

We also measured ALDH activity in a set of seven stocks heterozygous for second chromosome deletions. Three of the deletions covered CG3752, a putative ALDH structural locus identified by the *Drosophila* genome project (Flybase Consortium, 2003), while the remaining four were in other regions of the chromosome. CG3752 was chosen for initial investigation after a BLASTP search using human ALDH2 as a query identified it as the gene with the highest similarity to the latter in the *Drosophila* genome. The two proteins are similar in length (517 and 520 amino acids, respectively) and share 69% amino acid identity over 501 residues. Males of each deletion stock were crossed to virgin females of a stock isogenic for a *b c sp* second chromosome (Lindsley and Zimm, 1992), and wild-type (*i.e.,* deletion-bearing) progeny were used for ALDH assays.

Ethanol susceptibility assays

Because the HE populations have not previously been described, we report results of comparisons between them and the R populations in egg-to-adult survival, development time, and adult survival in the presence of ethanol. All flies were reared for two generations on regular food at a moderate density (4–5 pairs per shell vial, or 15 pairs per bottle) before being used for the comparisons (as well as for the enzyme assays described below). Larval and adult assays were conducted approximately 20 and 75 generations, respectively, after the HE populations were established. For the larval fitness assays, females were first allowed to lay eggs for 3–4 hours on apple juice-agar laying caps (Fry, 2001) supplemented with 12% ethanol (the latter was used to induce ethanol tolerance in the hatchling larvae; see Fry, 2001). Agar pieces with 50 eggs were placed in 28 mm diameter vials containing 10 ml of medium supplemented with 16% ethanol. Vials were set up on eight days (blocks), with four vials per population per block. Emerging flies were counted daily. For each vial, the proportion surviving, and the average times to emergence of males and females, were calculated.

Adult fitness assays were conducted by placing single-sex groups of 10 3–5 day old flies in 25 mm diameter vials containing a cotton plug to which 1 ml of a solution containing 15% (*v/v*) ethanol and 3% sucrose (*w/v*) had been added. Eight vials per sex and population were set up. Vials were sealed with corks, and flies checked twice daily. For each vial, the number of hours until half the flies had died (LT50) was estimated. Mortality in vials containing sucrose solution without ethanol was negligible.

Enzyme assays

Enzyme assays were performed approximately 100 generations after the HE populations were established. The extraction procedure followed Heinstra *et al.* (1989), except that separate sex groups of 25 flies were ground in 500 μl of cold extraction buffer. The supernatant was stored at −70°C until use. For the ALDH assay, 200 μl of supernatant was added to 1 ml of a reaction mixture containing 3.6 *mM* acetaldehyde, 1 *mM* NAD^+, and 0.2 *M* pyrazole in 0.1 *M* sodium pyrophosphate buffer, pH 8.5. Pyrazole was used to eliminate interference from ADH activity (Heinstra *et al.*, 1989). Absorbance was measured at 340 nm in 0.5 cm path-length cuvettes using an Ultrospec 2100 spectrophotometer (Amersham Pharmacia) programmed to take readings at one minute intervals for 20 minutes. For the ADH assay, 50 μl of supernatant was added to 1.15 ml of reaction mixture containing 1 *mM* NAD^+ and 0.1 *M* ethanol in 0.1 *M* sodium pyrophosphate buffer, pH 8.5. Absorbance was measured as above except for only three minutes (the increase in absorbance was linear over 20 minutes in the ALDH assays, but slowed noticeably after 3 minutes in the ADH assays, presumably because the reaction catalyzed by ALDH is irreversible, whereas that catalyzed by ADH is not). In both assays, the background change in absorbance was <5% that with substrate present, and varying the amount of fly supernatant resulted in proportional changes in the measured activity.

Because the spectrophotometer held eight cuvettes, each set of measurements (''block'') consisted of one sample from each population, with the sexes in different blocks. A total of five blocks were measured for each enzyme and sex. Total protein concentration of each supernatant was measured using a kit from Sigma (Micro Lowry method, Peterson's modification, direct protocol). Data were analyzed using the MIXED procedure in SAS (Littell *et al.*, 1996), with selection regime, sex, and the regime × sex interaction as fixed effects, and replicate population (nested within regime), block, sex × replicate, sex × block, and block × regime as random effects. Protein concentration was included as a covariate.

The procedure and analysis for the deletion stocks were similar, except there were only two blocks per sex.

RESULTS

Fitness assays

The two HE (''high ethanol'') populations had substantially higher egg-to-adult survival and faster development on medium supplemented with 16% ethanol than the two R (''regular food'') populations (Fig. 1). In addition, adults from the HE populations sur-

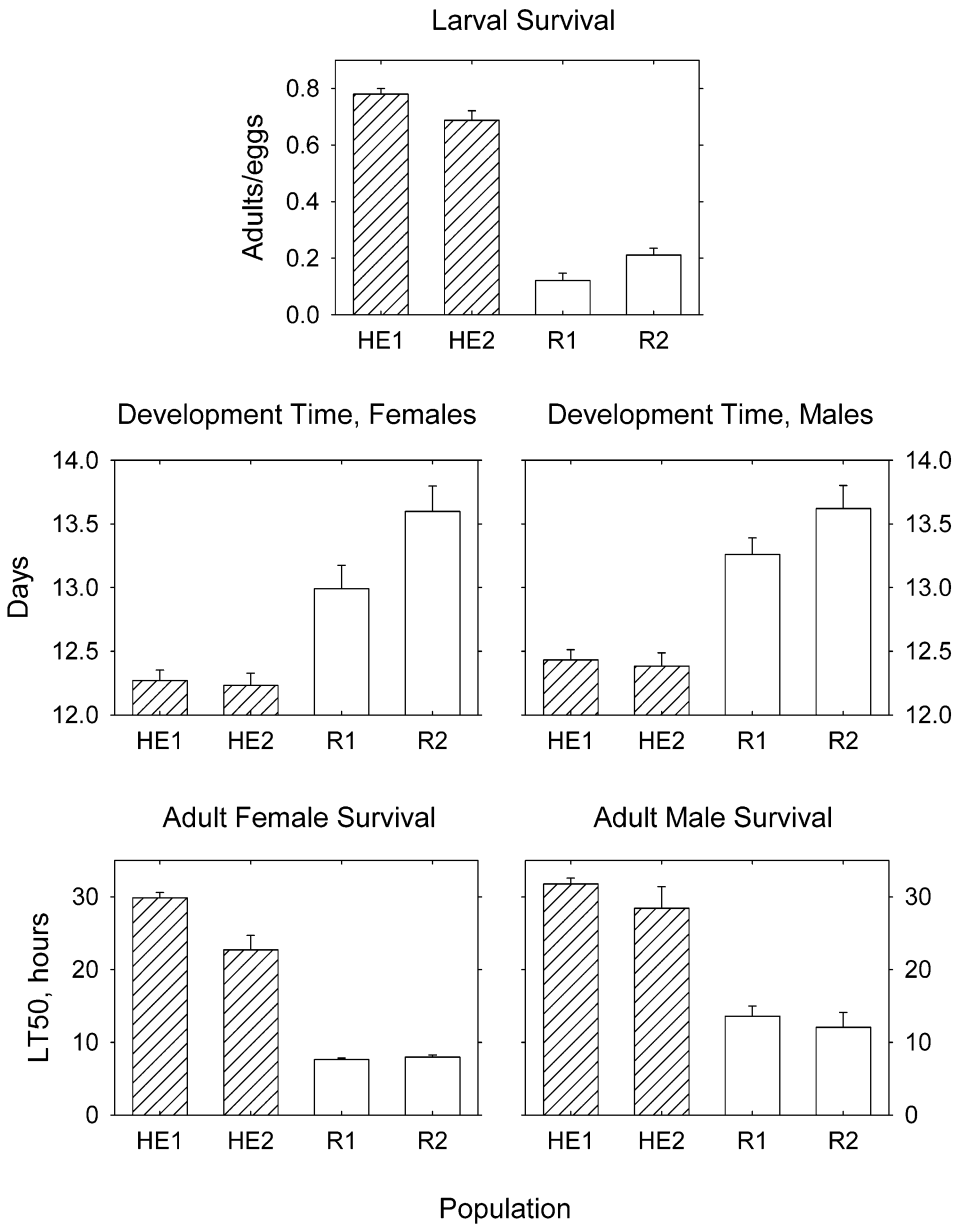

FIG. 1. Larval survival, development time, and adult survival of the HE (selected) and R (unselected) populations in the presence of ethanol. Means and SEMs are shown.

vived more than twice as long in vials containing a 15% ethanol solution than adults from the R populations (Fig. 1). *t*-tests comparing means of the HE populations with means of the R populations are in each case significant ($P \leq 0.04$ one-tailed).

ALDH and ADH activities in selected and unselected populations

ALDH activity showed a highly significant ($P = 0.004$) interaction between selection regime and sex (Table 1), indicating that the effect of selection regime differed between the sexes. An analysis of females alone shows a significant effect of selection regime ($F_{3,3.86} = 9.39$, $P = 0.03$), with activity increasing from the least (R) to the most (HE) ethanol-tolerant populations (Fig. 2). Among males, there was no significant effect of selection regime ($F_{3,4.06} = 2.13$, $P = 0.24$), although the HE populations had higher mean activities than the R populations (Fig. 2; this difference is significant at $P = 0.04$ in a one-tailed contrast).

There was a significant effect of selection regime on ADH activity, with no sex by regime interaction (Table 1). The R populations had lower ADH activity than populations from the other three regimes, with little variation among the latter (Fig. 3).

After adjusting for protein concentration, males dis-

TABLE 1. *Results of mixed-model analysis of ALDH and ADH activities.*

Effect	ALDH			ADH		
	F	df_{num}, df_{den}	P	F	df_{num}, df_{den}	P
Selection regime	4.45	3,3.97	0.092	10.12	3,3.99	0.025
Sex	27.72	1,28.7	<0.0001	31.28	1,20.4	<0.0001
Regime × sex	4.96	3,59.6	0.0039	0.64	3,4.04	0.63
Protein concentration	69.46	1,67.9	<0.0001	80.27	1,61.6	<0.0001

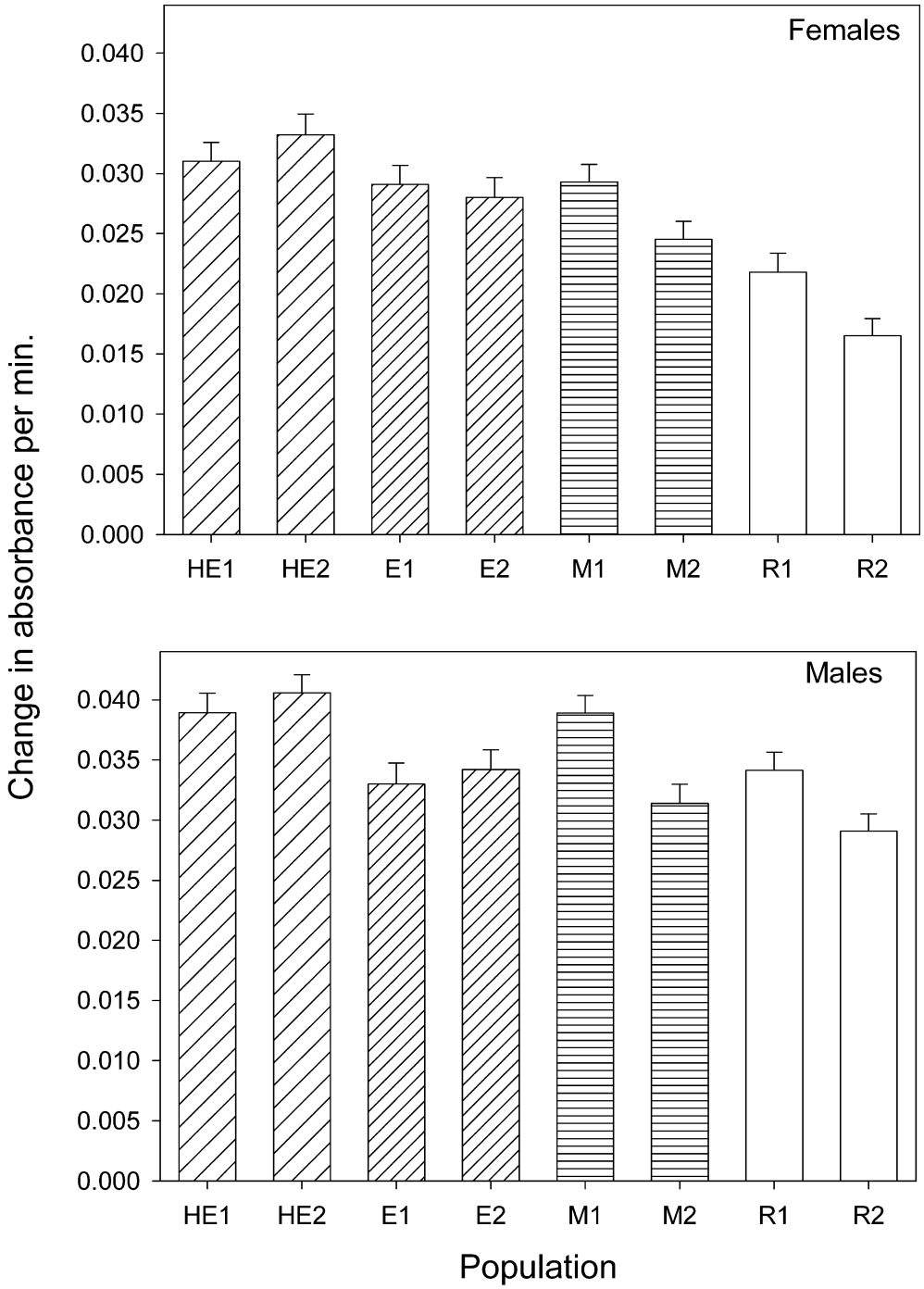

FIG. 2. Aldehyde dehydrogenase activity in the eight populations. Least-square means (adjusted for protein concentration) and standard errors are shown.

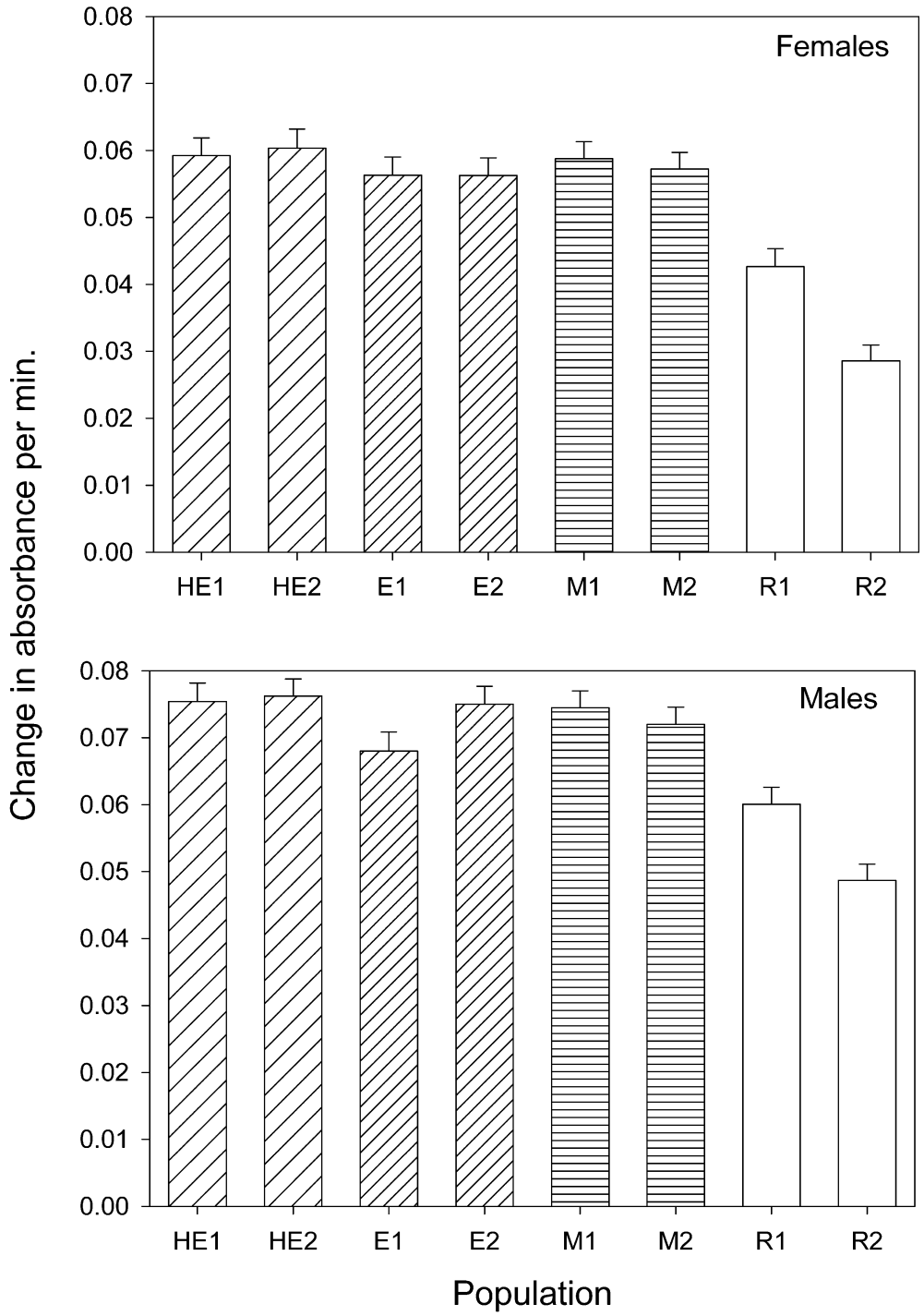

FIG. 3. Alcohol dehydrogenase activity in the eight populations. Least-square means (adjusted for protein concentration) and standard errors are shown.

played significantly higher activity of both enzymes than females (Table 1, Figs. 2, 3). Mean protein concentrations in the ALDH and ADH assays, respectively, were 929 and 232 μg/ml for females, and 642 and 160 μg/ml for males, reflecting the larger size of females and the greater amount of supernatant used in the ALDH assays. There was no effect of selection regime on protein concentration (analysis not shown).

ALDH activity of deletion stocks

Heterozygotes for deletions overlapping CG3752, a putative ALDH structural gene, had on average only 40% of the ALDH activity of heterozygotes for deletions not overlapping CG3752 (Table 2). An analysis similar to that in Table 1 shows the difference to be highly significant ($F_{1,4.63} = 150.5$, $P < 0.001$).

TABLE 2. *ALDH activity in deletion stocks.*

Genotype	No. copies of CG3752	Break 1[a]	Break 2[a]	ΔABS/min[b]	SE
Df(2L)N22-3/*b c sp*	1	30A1-2	30D1-2	0.0143	0.00197
Df(2L)N22-5/*b c sp*	1	29D1-2	30C4-D1	0.0127	0.00196
Df(2L)N22-14/*b c sp*	1	29C1-2	30C8-9	0.0117	0.00216
Df(2L)GpdhA/*b c sp*	2	25D7-E1	26A8-9	0.0354	0.00197
Df(2L)TW1/*b c sp*	2	38A7-B1	39C2-3	0.0298	0.00216
Df(2R)E2/*b c sp*	2	57B1	57B13-14	0.0323	0.00196
Df(2L)S2/*b c sp*	2	21C6-D1	22A6-B1	0.0353	0.00200

[a] Cytological break points. CG3752 is at 30B1.
[b] Least-square means from SAS.

DISCUSSION

In this study, *D. melanogaster* populations maintained on ethanol-supplemented media evolved higher activity of both ADH and ALDH. Increased ADH activity in the selected lines was observed in both sexes, whereas a clear increase in ALDH activity was observed only in females. Nonetheless, our results give the first evidence of which we are aware that genetic variation in ALDH activity can contribute to variation in ethanol tolerance in *Drosophila* populations.

We have also tentatively identified the locus responsible for most of the measured ALDH activity. Genotypes with one copy of CG3752, located on the second chromosome, had slightly less than half the ALDH activity than genotypes with two copies (Table 2). We are currently attempting to create a null mutation in this gene, using a stock with a *P*-element insert located near the transcription start site (Flybase Consortium, 2003). This insert does not disrupt the coding region, and has no detectable effect on ALDH activity (C.B. and J.D.F., unpublished data), but it should be possible to mobilize it to create additional mutations. The availability of ALDH-null or hypomorphic mutations will pave the way for a greater understanding of the importance of this enzyme in tolerance and utilization of ethanol in *Drosophila*.

We are also investigating whether the populations maintained on ethanol-supplemented media showed genetic changes at CG3752. Our preliminary results (W.C.S. and J.D.F., unpublished data) indicate that an amino-acid replacement substitution, rare in the unselected populations, increased in frequency in all of the selected populations. We are investigating whether the substitution directly affects ALDH activity, and whether the frequency of the high-activity variant increases with latitude, as is the case for *Adh.* A particularly interesting possibility is that CG3752 variants will interact with *Adh* alleles in determining fitness in the presence of ethanol. The combination of the high-activity *Adh-F* allele and a low activity ALDH-coding allele should be particularly disadvantageous, due to the rapid accumulation of acetaldehyde. Such an epistatic interaction could conceivably explain why *Adh-F* allele frequency increased in response to ethanol selection in some studies (Gibson, 1970; Bijlsma-Meeles and Van Delden, 1974; Cavener and Clegg, 1978) but not others (Gibson *et al.*, 1979; Oakeshott *et al.*, 1983, 1984).

Measurements of the flux of ethanol into lipid in different ADH genotypes have given evidence that ADH is the rate-limiting enzyme in larvae, but not adults (reviewed in Geer *et al.*, 1993; Heinstra, 1993). If this is true, we might expect variation in ALDH activity to have a greater effect on flux of ethanol into lipid in adults than larvae. The availability of mutant and natural alleles differing in ALDH activity should allow this prediction to be tested. It should be noted that even if variation in ALDH activity has little effect on net flux to ethanol in larvae, it could still affect larval fitness, for example by modulating acetaldehyde concentrations in mitochondria.

Another question which needs to be investigated is whether variation in ALDH activity among *Drosophila* species parallels variation in ethanol tolerance, as is the case for ADH activity. Garcin *et al.* (1986) compared developmental profiles of ALDH activity in a *D. simulans* strain and a *D. melanogaster* strain; the *melanogaster* strain had greater adult activity, but otherwise the two profiles were remarkably similar. It is not clear, however, whether the concentration of pyrazole they used (0.4 *mM*) was sufficient to inhibit all ADH activity (Moxon *et al.*, 1985). In any case, more strains and species need to be studied.

The results reported here give further evidence that ALDH plays a significant role in the ability of *D. melanogaster* to utilize resources high in ethanol. The contributions of variation in ALDH activity to the ethanol tolerance cline in *D. melanogaster*, and to the wide variation in ethanol tolerance among *Drosophila* species, remain to be elucidated.

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