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The alcohol dehydrogenase polymorphism of *Drosophila melanogaster* in relation to environmental ethanol, ethanol tolerance and alcohol dehydrogenase activity

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Ethanol levels in *Drosophila* breeding sites were higher in a winery storing fortified wines than in nearby grape pressings or in orchard fruits. The relative abundance of *D. simulans* to *D. melanogaster* was negatively correlated with ethanol levels. In *D. melanogaster* there were no significant differences in Adh^F frequency between the orchard and winery populations. The ethanol tolerance of wild caught *D. melanogaster* males paralleled the levels of ethanol in the breeding sites but *Adh* alleles and ethanol tolerance segregated largely independently of each other. Levels of ADH activity were positively associated with the ethanol tolerance of the different populations and with levels of ethanol in the breeding sites, but it is argued that the ethanol levels are not causative. Flies from inside the winery had higher ADH levels due mainly to greater amounts of ADH-F. The difference in activity persisted for at least one generation in the laboratory. After ten generations of laboratory culture the differences in ethanol tolerance were still present but there were no significant differences in ADH activity.

INTRODUCTION

The adaptive significance of enzyme polymorphisms in natural populations remains a controversial subject in the absence of unambiguous evidence for the mechanisms of selection (Hedrick *et al.*, 1980). The alcohol dehydrogenase (ADH, EC 1.1.1.1.) polymorphism in *Drosophila melanogaster* provides a good example of these difficulties (see reviews in McDonald, 1983, and Zera *et al.*, 1983).

Attempts to identify selective mechanisms maintaining the *Adh* polymorphism have utilised laboratory experiments and field studies on natural populations (see reviews in Clarke, 1975; Gibson, 1982; Van Delden, 1983). The laboratory experiments, focussing on the effects on *Adh* frequencies of variation in the concentration of a presumed ADH substrate, ethanol, have given conflicting results. Those single or multi-generation fitness experiments which used inbred lines, or material that had previously been maintained in the laboratory for a number of generations, generally showed that Adh^F (which produces more ADH than Adh^S, Gibson, 1972, Lewis and Gibson, 1978, Maroni, 1978, McDonald *et al.*, 1980 and Maroni *et al.*, 1982) increased in frequency in cultures maintained with media supplemented with ethanol greater than 6 per cent (v/v) (*e.g.*, Gibson, 1970). These results have led some workers to argue that heterogeneity in ethanol levels is involved in the maintenance of the *Adh* polymorphism. However, this argument is difficult to sustain in the face of results from similar experiments but using outbred, or freshly captured material, which have not demonstrated an increase in Adh^F (see discussion in Gibson and Oakeshott, 1982, Oakeshott *et al.*, 1984 and Barbancho *et al.*, 1987). It has also been shown that artificial selection for increasing tolerance to ethanol in populations segregating *Adh* alleles does not necessarily lead to an increase in Adh^F frequency (Gibson *et al.*, 1979).

Studies on natural populations comparing *Adh* frequencies inside and outside buildings processing and storing wine, and hence habitats assumed to differ in ethanol levels, have also given conflicting results. McKenzie and Parsons (1972; 1974) found no differences in *Adh* frequencies at

Chateau Tahbilk in Victoria, Australia, and neither did Marks *et al.* (1980) at a Sonoma Valley winery in California, nor Gibson *et al.* (1981) at seven Australian wineries. But Briscoe *et al.* (1975) and Hickey and McLean (1980) reported significantly higher frequencies of Adh^F inside wineries in Spain and Canada compared with habitats outside, and argued that the differences resulted from higher levels of ethanol inside wineries. This conclusion was not supported by data of Gibson *et al.* (1981) which showed that average ethanol concentrations in winery habitats (except those associated with fortified wines) did not exceed those found in decaying fruits and vegetables in orchards and gardens.

Some of the most consistent evidence for a relationship between ADH activity and ethanol tolerance derives from inter-specific comparisons. In particular *D. simulans*, with lower ADH levels than the Adh^S homozygotes of *D. melanogaster*, has much lower ethanol tolerance and is rare inside wineries. Further Libion-Mannaert *et al.* (1976) and Parsons and Stanley (1981) have shown that the utilisation of ethanol as an energy source correlates with ADH activity within and between species and Ziolo and Parsons (1982) have argued that tolerance to ethanol is an ecologically significant phenotype of Drosophilids.

Interpretation of the data on the *Adh* polymorphism in natural populations is made difficult because none of the studies have directly compared both the ethanol tolerance and the ADH activities of *Adh* genotypes in populations exposed to different measured levels of ethanol. Thus the conflicting results could be explained by assuming, for example in cases where no differences in gene frequency were detected, that ethanol levels did not differ between habitats or that there were no differences in ADH activity between *Adh* genotypes in natural populations.

To overcome these difficulties in elucidating the role of ethanol tolerance in the maintenance of the *Adh* polymorphism we have investigated newly captured *D. melanogaster* and *D. simulans* from populations inhabitating environments with a range of ethanol levels from less than 1 per cent to more than 9 per cent.

MATERIALS AND METHODS

All of the experiments, except where otherwise stated, were carried out on newly captured flies from in and around the All Saints winery near Rutherglen, Victoria, Australia. The All Saints winery specialises in fortified wines (up to 20 per cent v/v ethanol) which are stored above ground in oak casks. Part of the same building is used for the processing of wine and contains open fermentation vats. The flies were collected between February and April in 1981 and 1982 from the following four types of habitat:

(a) decaying fruits (mainly peaches, pears and plums) in an orchard approximately 6 km from the All Saints winery.
(b) dumps of grape skins (must) in the vineyard 400 m from the winery.
(c) the surface and edges of open fermentation vats sited just inside the winery but close to a large entrance.
(d) leakage from barrels containing fortified wines.

At all these sites flies were swept by net, or allowed to emerge from samples of the material in which they were breeding. In all respects investigated, flies obtained by these two methods of sampling gave consonant results.

Flies were maintained in the laboratory on standard media containing 10 g agar, 50 g glucose, 26 g sucrose, 50 g maize meal, 22·5 g wheat germ and 6 g yeast made to 1 litre with water with 12·5 ml of acid mix comprising 42 per cent proprionic acid and 4 per cent orthophosphoric acid.

The levels of ethanol were measured as previously described (Gibson *et al.*, 1981) in samples taken from sites at which *D. melanogaster* had been shown to be breeding in each of the four types of habitat. The samples, each weighing approximately 1 g, were put into scintillation vials which were immediately sealed with a rubber septum and then frozen in liquid nitrogen to prevent loss of ethanol. Ethanol levels were assayed by the "head space" method of Brien and Loomis (1978), in a Hewlett Packard gas chromatograph (Gibson *et al.*, 1981).

Tolerance to ethanol was measured in each sex by keeping 17 cohorts, each of 20 flies, in 300 ml culture bottles with 50 ml of standard media containing 9 per cent (v/v) ethanol and scoring the number of flies alive after six days. This level of ethanol was used because laboratory experiments indicate that concentrations above 6 per cent (v/v) provide a metabolic cost to *D. melanogaster* (Parsons and Stanley, 1981) and, at least in inbred material, can lead to an increase in the frequency of Adh^F (Van Delden, 1983). The flies used in these tests were collected on emergence from random samples of up to 200 ml of material taken from breeding sites in each of the four types of

habitat, and similar numbers of five to seven day old flies were used from each of the samples from a particular habitat. Extra flies from each habitat were typed for *Adh* genotypes (see below) and these data are included in table 3 as the March 1981(a) samples.

Adh genotypes were scored after electrophoresis of single fly extracts on cellulose acetate membranes using the method described by Lewis and Gibson (1978). The frequency of a third allele Adh^{FChD} was also scored in the flies collected in 1981 and 1982 using the technique of Wilks *et al.* (1980). However, its frequency was always found to be less than 5 per cent in these populations and it was not scored in the ethanol tolerance experiments or in the single fly assays. The ADH electrophoresis patterns were used to distinguish *D. simulans* females (post mortem in the tolerance tests) from *D. melanogaster*; the males of the two species were classified by their external genitalia and sorted prior to the experiments. The 1981 and 1982 collections were also scored for sn-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8.) *Gpdh* genotypes after electrophoresis on cellulose acetate membranes (Gibson *et al.*, 1986).

ADH activity was measured in crude homogenates of 20 male flies as previously described (Gibson *et al.*, 1980). Unless otherwise stated the assays were made on flies collected in the wild from the four habitats. Assays were separately made with 2-propanol and ethanol as substrate but as there was a correlation of 0·93 between the two sets of activities we report the data obtained with 2-propanol. In some experiments ADH was assayed in single male flies also taken directly from the natural populations. For these assays each fly was weighed and put into a micro-centrifuge tube held in liquid nitrogen and then ground up by a perspex pestle shaped to fit the micro-centrifuge tube. Buffer was added to the frozen fly in proportion to its weight (200 µl/mg). The samples were centrifuged at 10,000 g for 3 mins and the supernatant was assayed for ADH (Gibson *et al.*, 1980). The pellet was used to prepare a sample for the electrophoretic determination of the fly's *Adh* genotype. In most cases the flies' ADH activities and tolerance were measured within 48 hrs of capture during which time the flies were held on standard laboratory culture media.

The amount of ADH protein in crude extracts was assayed by radial immunodiffusion (Mancini *et al.*, 1965) as modified by Lewis and Gibson (1978) and using the antisera they describe, which were produced in rabbits.

RESULTS

The ethanol levels detected in *Drosophila* breeding sites in the 1981 and 1982 samples are shown in table 1. There was significant heterogeneity in ethanol content between the four kinds of habitat in and around the All Saints winery. The highest mean level was found in leakages from barrels of fortified wines inside the winery (3·2 ± 0·62 per cent v/v) and this was significantly higher ($t_{52} = 3·9$, $p < 0·001$) than the mean (2·3 ± 0·72 per cent v/v) in the fermentation vats inside the winery but close to an entrance. At breeding sites outside the winery the mean ethanol level was 1·21 ± 0·67 per cent (v/v) in grape pressings and 1·62 ± 0·34 per cent (v/v) in the orchard. These values were not significantly different, but they were lower than the levels in the fermentation vats ($t_{53} = 3·7$, $p < 0·001$).

D. simulans was at very low frequency in collections from inside the winery and was not found in the samples from the fermentation vats (table 2). The frequency was lower in the grape skin dump than in the orchard ($\chi^2_1 = 24·97$, $p < 0·001$). Nevertheless, *D. simulans* was found among flies that emerged from samples with a mean ethanol level of 5·84 per cent (v/v) (table 1).

In *D. melanogaster* the proportion of female flies was higher in collections from inside the winery and at the fermentation vats and the excess was higher than at the grape skin dump ($\chi^2_1 = 13·09$, $p < 0·001$) which in turn was higher than at the orchard ($\chi^2_1 = 60·1$, $p < 0·001$). In the *D. melanogaster* and *D. simulans* samples there was a higher frequency of males in the orchard collections. A similar distribution of *D. melanogaster* was reported by McKenzie (1974) at another Victorian winery, Chateau Tahbilk.

All samples of *D. simulans* were monomorphic for a single electrophoretically detectable *Adh* allele and for a *Gpdh* allele, as was expected from other population surveys (Berger, 1970).

Three electrophoretically distinguishable alleles (Adh^F, Adh^{FChD}, Adh^S) were segregating at the *Adh* locus in all the *D. melanogaster* samples (table 3). The frequency of Adh^F varied between 0·65 and 0·69 but there was no evidence for heterogeneity in *Adh* genotypes between any of the populations sampled in 1981 (a, $\chi^2_3 = 0·16$, $p > 0·05$; b, $\chi^2_2 = 0·02$, $p > 0·05$) or in 1982 ($\chi^2_3 = 1·14$, $p > 0·05$).

In view of the evidence obtained by Cavener and Clegg (1978, 1981) that *Adh* genotypes interact with *Gpdh* genotypes under high ethanol stress in

Table 1 Levels of ethanol in *D. melanogaster* breeding sites. Those samples marked † include ones from which *D. simulans* also emerged; (a), (b) and (c) represent separate barrels of wine or separate piles of grape pressings

Type of habitat		Number of samples	Ethanol (% v/v) Mean ± standard error	Range
Inside winery, leakage from barrel containing				
muscat	(a)	2	4·49 ± 0·05	4·41–4·56
	(b)	5	5·77 ± 3·01	1·15–9·17
sherry	(a)	3	1·30 ± 0·12	1·09–1·51
	(b)	2	3·22 ± 0·74	1·96–4·47
	(c)	4	2·33 ± 1·05	0·12–5·06
port	(a)	3	2·79 ± 2·43	0·35–7·64
	(b)†	8	4·81 ± 0·86	1·63–7·79
	(c)†	10	0·71 ± 0·77	0·12–2·52
Open fermentation vats				
liquid in vat	(a)	2	2·54 ± 0·06	2·48–2·60
	(b)	2	4·86 ± 0·31	4·55–5·18
	(c)†	2	5·84 ± 0·06	5·78–5·89
grape skins	(a)	2	2·88 ± 0·10	2·78–2·97
	(b)†	9	1·46 ± 0·95	0–4·26
Outside winery				
discarded pressings	(a)†	4	0·19 ± 0·01	0–0·46
	(b)†	2	0·08 ± 0·04	0·03–0·14
	(c)	4	2·88 ± 2·04	1·11–4·65
20 cm under surface of grape pressings	(c)	8	1·89 ± 1·58	0·31–5·45
Orchard				
pears	†	6	1·92 ± 1·31	0·23–3·86
plums	†	14	1·36 ± 1·52	0–4·35

Source	Analysis of variance df	M.S.	F
Between habitats	3	16·2	4·4**
Within habitats	88	3·7	

** $p < 0·01$.

Table 2 Proportions of *D. simulans* and *D. melanogaster* and their sex ratios, ♀/♂, in collections made in March 1982

	Orchard	All Saints		
		Grape skin dump	Fermentation vats	Inside winery
Total flies scored	830	620	522	560
Percentage *D. simulans*	59·1	34·1	non detected	0·3
Sex ratio *D. simulans*	0·34*	0·54	—	—
Sex ratio *D. melanogaster*	0·28*	0·55	0·64*	0·66*

* Significantly different to 0·5, $p < 0·001$.

Table 3 *Adh* allele frequencies in *D. melanogaster* in a Rutherglen orchard and at All Saints

					All Saints							
		Orchard			Grape skin dump			Fermentation vats			Inside winery		
Collection		*n*	Adh^F	Adh^{FChD}	*n*	Adh^F	Adh^{FChD}	*n*	Adh^F	Adh^{FChD}	*n*	Adh^F	Adh^{FChD}
March 1981	(a)	324	0·68	0·01	264	0·67	0·02	260	0·66	0·02	258	0·68	0·02
								224	0·68	0·01			
	(b)	—	—	—	190	0·67	0·03	198	0·67	0·02	196	0·65	0·04
								196	0·68	0·04			
March 1982		389	0·67	not scored	460	0·69	not scored	192	0·69	not scored	464	0·66	not scored

n = number of genes scored.

laboratory experiments, *Gpdh* electrophoretically distinguishable alleles were scored in the flies, which were also scored for *Adh*, from the grape skin dump, the fermentation vats and the barrels. There was no heterogeneity in *Gpdh* frequency (mean $0{\cdot}74 \pm 0{\cdot}03$) between sites ($\chi^2_4 = 4{\cdot}8$, $p > 0{\cdot}05$) or any evidence of gametic disequilibrium with *Adh* alleles ($R = 0{\cdot}13$, $p > 0{\cdot}05$), (Knibb, 1983).

Ethanol tolerance tests were carried out on flies from 26 of the breeding sites included in table 1. The average ethanol levels (per cent v/v) in these sites were, $1{\cdot}64 \pm 0{\cdot}28$ in ten samples from the orchard, $1{\cdot}26 \pm 0{\cdot}68$ in four samples from the grape skin dump, $2{\cdot}17 \pm 0{\cdot}71$ in four samples from around the edges of the fermentation vats and $3{\cdot}16 \pm 0{\cdot}62$ in eight samples from separate leakages from barrels of fortified wine (between habitats, $F_{3/22} = 6{\cdot}2$, $p < 0{\cdot}01$). There was a significant difference in ethanol tolerance between habitats (table 4) although the flies from fermentation vats and inside the winery were similar ($t_{32} = 0{\cdot}75$, $p > 0{\cdot}05$) as were those from the grape pressings and the orchard ($t_{32} = 1{\cdot}3$, $p > 0{\cdot}05$). The tolerances of male and female flies from inside the winery and from the fermentation vats were significantly higher than that of flies from the orchard and the grape skin dump (for males $t_{66} = 4{\cdot}3$, $p < 0{\cdot}001$, for females, $t_{66} = 4{\cdot}1$, $p < 0{\cdot}001$). Overall there was no significant sex difference, nor sex × habitat interaction, in tolerance. A smaller study of ethanol tolerance carried out in the same way but using net caught flies from around the same breeding sites gave consonant results (between habitats, $F_{3/42} = 0{\cdot}9$, $p < 0{\cdot}05$).

Tolerance tests were also carried out on *D. simulans* collected in the orchard (insufficient flies were available from the other habitats). The average mortality was over 90 per cent using the same test conditions as for *D. melanogaster*.

Table 4 Mean ethanol tolerance (% survival with standard errors in angles) in *D. melanogaster* collected at different breeding sites

Sex	Orchard	Grape skin dump	Fermentation vats	Inside winery
Males	$23{\cdot}2 \pm 1{\cdot}9$	$24{\cdot}7 \pm 1{\cdot}6$	$42{\cdot}1 \pm 1{\cdot}8$	$54{\cdot}4 \pm 1{\cdot}8$
Females	$33{\cdot}2 \pm 2{\cdot}2$	$35{\cdot}0 \pm 2{\cdot}4$	$36{\cdot}8 \pm 2{\cdot}2$	$47{\cdot}3 \pm 1{\cdot}9$

Analysis of variance of angularly transformed data

Source	df	M.S.		*F*
Habitats	3	1355·9		8·4***
Sex	1	118·6	($F_{96/1}$)	1·4 ns
Replicates	16	56·6	($F_{96/16}$)	2·8 ns
Sex × Habitats	3	318·9		2·0 ns
Sex × Replicates	16	53·2	($F_{96/16}$)	3·0 ns
Error	96	161·2		
Total	135			

*** $P < 0{\cdot}001$.

In order to test for a differential effect of ethanol on survival of the *D. melanogaster Adh* genotypes all the male flies surviving after seven days in the ethanol tolerance tests, and a similar number from cohorts of male flies held on standard media for six days, were scored for *Adh* genotypes. As large samples of male flies (340) from each of the four habitats were initially placed on each type of media it was assumed that the initial *Adh* gene frequency on standard and ethanol media were the same. These data (table 5) show that there are no significant differences in gene frequencies between survivors on ethanol and standard media, whether the tested flies came from inside or outside the winery. Thus ethanol tolerance of adult male flies is not associated with *Adh* genotype in any of the four habitats.

In samples of male *D. melanogaster* caught in the wild and assayed within three days, there was significant heterogeneity in ADH activity between habitats (table 6), although activity was similar in flies from inside the winery and from the fermentation vats ($t_8 = 1{\cdot}65$, $p > 0{\cdot}05$) and between the grape skin dump and orchard samples ($t_8 = 0{\cdot}66$, $p > 0{\cdot}05$); the significant difference was between samples from the winery and those from outside ($t_{18} = 4{\cdot}12$, $p < 0{\cdot}001$). This heterogeneity in ADH activity was found again in samples taken from similar sites a year later in 1982 (table 6) although in these samples the overall mean activity was higher than in 1981.

The 1981 and 1982 wild caught *D. melanogaster* samples from the orchard, grape skin dump, fermentation vats and inside the winery were scored for the amount of ADH protein. There was a correlation of 0·89 between ADH activity and the amount of ADH protein. In analyses of variance of these data both the differences between habitats ($F_{3/32} = 18{\cdot}7$, $p < 0{\cdot}001$) and between years ($F_{1/32} = 10{\cdot}2$, $p < 0{\cdot}001$) were significant.

From each of the four sites five cultures of 40 females from the 1981 samples were maintained on standard laboratory food for one generation, then samples of F_1 male flies (6–8 days from emergence) were assayed for ADH activity. These data (table 6) show that the differences in ADH activity between the wild caught flies from the four habitats persist in the progenies cultured in the laboratory. The amount of ADH protein also differed between the habitats ($F_{3/16} = 8{\cdot}6$, $p < 0{\cdot}01$).

Further assays of ADH activity and of ethanol tolerance were made when the flies collected on the grape pressings and from the barrel leakages had been maintained for ten generations on standard laboratory media at 22°C. Five separate cultures were maintained and assayed from each of the two habitats. In these tests the flies derived from collections around leaking barrels remained more tolerant to ethanol ($t_8 = 6{\cdot}5$, $p < 0{\cdot}001$) than those from the grape pressings (table 7) but the tolerances were less than the levels observed in newly captured material (grape skin dump $t_8 = 3{\cdot}1$, $p < 0{\cdot}005$; inside the winery $t_8 = 2{\cdot}6$, $p < 0{\cdot}05$). It is noteworthy that the frequencies of Adh^F had not diverged significantly either in the two set of cultures (χ^2_1, $= 0{\cdot}36$, $p > 0{\cdot}05$) or from the frequencies observed in wild caught material (χ^2_1, $= 0{\cdot}36$, $p > 0{\cdot}05$) (cf. tables 3 and 7).

Samples of *D. simulans* males from the orchard and from the grape pressings were assayed for ADH (insufficient flies were available from the collections at the other sites). These data (table 6) show that ADH activity in *D. simulans* is about a fifth of that in the *D. melanogaster* orchard population. It is interesting that *D. simulans* from the grape pressings have significantly higher ADH activity ($t_4 = 2{\cdot}8$, $p < 0{\cdot}05$) than those from the orchard, and significantly higher amounts of ADH protein ($t_4 = 2{\cdot}9$, $p < 0{\cdot}05$) as measured using the antiserum prepared against *D. melanogaster* ADH.

To test whether there were significant differences in ADH activity between flies of the different *Adh* genotypes one hundred *D. melanogaster* male flies collected around barrel leakages and a hundred collected from the grape pressings were assayed individually for ADH activity and scored

Table 5 Distribution of *Adh* genotypes among male flies surviving after seven days on standard media and on 9% ethanol media

Medium	Orchard			Grape skin dump			Fermentation vats			Inside winery		
	FF	FS	SS	FF	FS	SS	FF	FS	SS	FF	FS	SS
Laboratory media	43	41	8	48	45	9	63	36	7	43	47	9
9 per cent ethanol	49	43	6	53	50	6	58	45	8	55	47	8
χ^2_1	0·39 $p > 0{\cdot}5$			0·26 $p > 0{\cdot}5$			0·76 $p > 0{\cdot}1$			0·42 $p > 0{\cdot}2$		

Table 6 Alcohol dehydrogenase activity (units/mg live weight) in male flies ± standard errors. For the *D. melanogaster* samples five (and for the *D. simulans* samples three) separately prepared extracts were assayed from each habitat. One unit of ADH activity is defined as an increase in absorbance at 340 nm of 0·001/min (equivalent to $1{\cdot}61\times10^{-4}$ moles NADH produced per min)

Collection	Orchard	Grape skin dump	Fermentation vats	Inside winery
D. melanogaster				
1981 wild caught	140·2±4·9	136·2±3·6	152·4±8·9	168·7±4·3
1981 laboratory reared one generation	141·1±5·2	133·7±2·7	148·5±3·5	163·8±3·8
1982 wild caught	149·3±4·7	145·1±3·9	163·7±5·0	208·2±3·6
D. simulans				
1982 wild caught	29·2 (1·1)	38·1 (3·0)	—	—

Analysis of variance

Source	df	M.S.	*F*
(a) Wild caught flies			
Habitats	3	4735	19·5***
years	1	2706	11·1**
Habitats × years	3	624	2·6 ns
Error	32	243	
(b) Laboratory reared flies			
Habitats	3	980	8·8**
Error	16	112	

** $p<0{\cdot}01$.
*** $p<0{\cdot}001$.

for ADH electrophoretic phenotype. Four points emerge from the results of these assays (table 8). First, *Adh* genotype frequencies do not differ significantly between sites ($\chi^2_2=0{\cdot}74$, $p>0{\cdot}05$); second, segregation of electrophoretically distinguishable alleles at the *Adh* locus accounts for the vast majority of variation in ADH activity as there is virtually no overlap in ADH activity between the three *Adh* genotypes (see also Anderson and Gibson, 1985). Third, there is a significant difference in ADH activity between flies from the two types of habitat. Fourth, although the habitats × genotype interaction is not significant inspection of the data (table 8) shows that the Adh^F homozygotes from inside the winery have significantly higher activity than Adh^F homozygotes from the grape skin dump ($t_{92}=2{\cdot}9$, $p<0{\cdot}01$).

Table 7 ADH activity and tolerance (± standard errors) to ethanol of male flies from the 1981 samples maintained for ten generations in the laboratory on standard media. Each value is the mean obtained from extracts of five separate cultures

	Grape skin dump	Inside winery
ADH activity	140·5±4·6	146·2±7·7
Ethanol tolerance (% surviving)	15·4±1·4	36·5±2·1
Adh^F frequency	0·71±0·05	0·69±0·07

Table 8 Alcohol dehydrogenase activities, with standard errors, in assays of single male flies (sample sizes in parentheses)

Source of flies	*Adh* Genotypes FF	FS	SS
Inside winery	189±5·1 (49)	113±4·5 (42)	57±5·5 (9)
Grape skin dump	166±6·3 (46)	114±4·4 (45)	56±3·3 (9)

Analysis of variance

Source	df	M.S.	*F*
Habitats	1	7,509	4·5*
Adh genotypes	2	155,838	92·3***
Habitats × genotypes	2	3,482	2·1 ns
Error	194		

* $p<0{\cdot}05$.
*** $p<0{\cdot}001$.

There are no significant differences in activity between either the Adh^S homozygotes ($t_{16}=0{\cdot}17$, $p>0{\cdot}05$) or between the Adh^F/Adh^S heterozygotes ($t_{85}=0{\cdot}18$, $p>0{\cdot}05$) from the two habitats. (We have found that the mean ADH activity in single fly assays is consistently lower than the value obtained from mass homogenates, possibly due to the differences in sample preparation). There was insufficient material remaining in these single fly extracts to measure the amounts of ADH protein, but in tests on similar samples there was a correlation of 0·84 between ADH protein amount and ADH activity in single fly assays.

In view of these results the ADH activities of flies which survive ethanol tolerance tests were investigated and compared with the activities of flies kept on control (standard) media. Extracts were made from four sets of 20 male flies taken from flies held on control media and from the survivors of the ethanol tolerance tests on samples from the grape skin dump and from inside the winery. These data (table 9) show that there is no significant difference in ADH activity in flies from the grape skin dump ($t_6=0{\cdot}54$, $p>0{\cdot}05$) or from inside the winery ($t_6=0{\cdot}61$, $p>0{\cdot}05$) compared with those kept on control media for six days. This shows that flies of any *Adh* genotype with higher than average ADH activity do not have higher than average survival in the tolerance tests. It also indicates that ADH activity in adult flies is not increased by exposing the flies to 9 per cent ethanol media for six days.

Table 9 ADH activities (± standard errors) in four separate assays of extracts of male flies from the grape skin dump and inside the winery which survive the tolerance tests or have been held on control media. Four separate extracts were assayed from each condition

	ADH activity	
Source of flies	Ethanol media	Control media
Inside winery	170·2 ± 5·6	167·9 ± 4·3
Grape skin dump	138·4 ± 3·7	141·5 ± 3·9

DISCUSSION

The data described here focus on natural populations of *D. melanogaster* and *D. simulans* which feed and breed in habitats heterogeneous for ethanol levels up to 9 per cent (v/v). The habitats compared therefore provide a range of ethanol environments to which genetic adaptation might occur. It is important to emphasise that the ethanol levels in some of these breeding sites are not dissimilar to those which have been used in media in laboratory cultures and in which increases over generations in the frequency of Adh^F, relative to Adh^S, have been reported (for reviews of these experiments see Gibson and Oakeshott (1982) and Van Delden (1983)).

Two observations support the notion that the *Drosophila* populations investigated in this study have adapted to the different environments. First, and most importantly, there is the evidence that in the wild caught *D. melanogaster* the extent of tolerance to 9 per cent ethanol in the media parallels the levels of ethanol detected in the natural breeding sites from which the flies were collected.

Second, the relative abundance of *D. simulans*, which is very much less tolerant to ethanol than *D. melanogaster* (Parsons and Stanley, 1981), is lower at breeding sites inside the winery which have relatively high levels of ethanol compared to sites outside. Distributions similar to this have been reported before (McKenzie, 1974; Marks *et al.*, 1980; Gibson *et al.*, 1981); however the causative role of ethanol levels has been questioned, and other factors associated with buildings might be involved (David, 1979; Rouault and David, 1982). In the present study *D. simulans* were collected from habitats with more than 5·7 per cent (v/v) ethanol, which is higher than the average level in leakages from barrels, so that ethanol levels *per se* may not be responsible for the virtual exclusion of *D. simulans* from the winery habitats.

The adult sex ratio differences observed between the winery and orchard samples of *D. melanogaster*, and which have been reported in previous studies of winery habitats (McKenzie, 1974), are not due to sex differences in ethanol tolerance and remain unexplained. It will be informative in future work to compare the sex ratio at emergence with the sex ratio in net caught adults in each type of habitat.

Turning to the genetic consequences in *D. melanogaster* of adaptation to different ethanol levels, the evidence shows that changes in the frequencies of *Adh* alleles are not involved. In agreement with three other studies (McKenzie and McKechnie, 1978; Marks *et al.*, 1980; Gibson *et al.*, 1981) the data for All Saints show that the distribution of *Adh* alleles and genotypes in *D. melanogaster* was homogeneous over all collections, whether taken inside or outside the winery. Contrary results were reported by Briscoe *et al.*

(1975) who observed significantly lower frequencies of Adh^F on a rubbish dump 1 km from a wine cellar in Spain, and by Hickey and McLean (1980) who found significantly lower Adh^F frequencies at sites more than 3 km from a Canadian winery. Hickey and McLean (1980) argued that sampling range might explain the differences between the data of Briscoe *et al.* (1975) and those of McKenzie and McKechnie (1978). However the present data, together with those described by Gibson *et al.* (1981), do not support this idea as the sites sampled encompass as wide a range of distances between winery and non-winery sites as the previous studies. Briscoe *et al.* (1975) reported that *D. melanogaster* were breeding in mats covering storage casks containing sherry with "12–15 per cent ethanol". However no other data on ethanol levels were given and it is possible that they were similar to those measured from leaking barrels of sherry at All Saints. It thus seems unlikely that levels of ethanol are implicated in the differences between the studies, although it remains possible that the mode of exposure to ethanol was important in bringing about the variation in Adh^F frequencies (Oakeshott and Gibson, 1981).

Our direct evidence from tests of the relative fitness of *Adh* genotypes in wild caught flies kept on 9 per cent ethanol media compared with those on standard media supports the gene and genotype frequency distributions observed in the All Saints habitats. Despite the high mortalities which occurred in these tests there were no significant differences in Adh^F frequency between survivors on ethanol media and those on standard media. Further, the survivors on ethanol media did not have higher ADH activity. Thus it seems clear that tolerance to 9 per cent ethanol in these wild caught flies, whether they are from inside or outside the winery, is independent of allozyme variants at the *Adh* locus. This result agrees with the observations of McKenzie and Parsons (1972) who investigated the genetic basis of ethanol tolerance in flies from the Chateau Tahbilk winery and found that variation at the *Adh* locus contributed little to variation in ethanol tolerance. It remains possible that flies are occasionally exposed to ethanol levels (above 9 per cent) which do impose measureable selective differences. However any effects of such sporadic selection on *Adh* frequencies would be unlikely to persist.

If variation at the *Adh* locus has only a minor role in adaptation to ethanol, then the evidence that ADH activity varies between habitats is intriguing. Between the four breeding sites of *D. melanogaster*, ethanol levels, ethanol tolerance and ADH activity are positively associated, although, within the population of each type of habitat, ethanol tolerance is independent of *Adh* genotype and of variation in ADH activity. These data do not rule out a role of ADH in ethanol tolerance but they show that the within population variation in ADH is not important for tolerance in *D. melanogaster*.

There is evidence from other studies for modifiers of ADH activity in *D. melanogaster* either linked or unlinked to the *Adh* locus (Ward and Hebert, 1972; Ayala and McDonald, 1980; Maroni *et al.*, 1982; Maroni and Laurie-Ahlberg, 1983) but in the present data, and in other populations studied (Anderson and Gibson, 1985), the main cause of the variation in ADH activity is differences among the three *Adh* genotypes. Thus our observation that ethanol tolerance is independent of segregation at the *Adh* locus within each population suggests that the association of differences in ADH activity and in ethanol tolerance between populations are not causally related. It remains possible that any variation in ADH activity due to modifier genes or environmental induction might have different physiological effects. For example modifiers may cause ADH to be expressed with a different tissue distribution which might have larger effects on tolerance than variation in level within the same tissue (Clarke and Whitehead, 1984).

Our conclusion that variation in ADH activity and ethanol tolerance are not causally related in this material is at variance with evidence derived from studies of inbred laboratory lines (Morgan, 1974; Thompson and Kaiser, 1977). Nevertheless it is in agreement with the more relevant data on ADH activity in lines selected for ethanol tolerance (Gibson *et al.*, 1979) which showed that no change in activity accompanied successful selection for ethanol tolerance.

The causes of the ADH activity differences between flies from different breeding sites are being investigated but four points must be taken into account in any explanation. First, although the differences in activity in *D. melanogaster* persisted after one generation of culture on standard laboratory media they were not detectable after 10 generations. Second, after 10 generations the association between ADH activity and ethanol tolerance in *D. melanogaster* was lost and only the differences in tolerance persisted. Third, the higher ADH activity in *D. melanogaster* from the barrel leakages compared with flies from the grape dump seems to be mainly ascribable to the activities of Adh^F/Adh^F homozygotes although this does not

completely account for the difference observed in assays of segregating cultures. Fourth, the ADH activities of *D. simulans* from the grape pressings are higher than those from the orchard collections even though our evidence shows that these two types of habitat do not differ significantly in ethanol levels.

Taken together these observations might be explained if there was genetic heterogeneity between breeding sites for electrophoretically cryptic *Adh* alleles, or modifiers, encoding different amounts of ADH. The electrophoretically cryptic allele Adh^{FChD} occurs at a frequency of less than 5 per cent in the All Saints populations and there is no evidence for heterogeneity between habitats. Studies comparing ADH activities in lines homozygous for four Adh^{FChD} alleles extracted from inside the winery with four alleles extracted from the grape skin dump have shown no significant difference in activity ($t_6 = 1{\cdot}3$, $p > 0{\cdot}05$). It thus seems unlikely that Adh^{FChD} contributes to the differences in activity between the habitats.

We have no quantitative data on the movement of flies between different parts of the winery but there are no obvious barriers to migration between the grape skin dumps, the fermentation vats close to the entrance to the winery and the wine storage barrels inside. Indeed, during the vintage period there is a daily traffic of grapes from the vineyard to the pressers just outside the winery building, and of must out to the dumps in the vineyard. However, the evidence that differences in ADH activity persist for at least one generation of laboratory culture suggests that migration between the winery habitats is in some way restricted, or alternatively, selective.

Other than in levels of ethanol, the orchard, grape pressings and winery breeding sites of *D. melanogaster* are likely to differ in the quantities and types of yeast and bacteria as well as in the concentrations of other nutrients. Clarke *et al.* (1979) demonstrated that the amount of yeast in laboratory cultures had large effects on ADH activity, and other substances likely to be present in natural habitats also affect ADH activity (Schwartz and Sofer, 1976; Papel *et al.*, 1979). McKechnie and Geer (1984) have shown that in axenic cultures with 0·5 per cent (w/v) sugar, relatively low concentrations of ethanol (2·5 per cent (v/v)) give rise to two-fold increases in larval ADH activity, at least in the Adh^S line with which they worked. Whatever the cause of the activity differences in the All Saints populations, the evidence is suggestive of differential regulation of Adh^F and Adh^S alleles, although it is surprising that the Adh^F/Adh^S heterozygote shows no effect. It will be important to test whether the same effect occurs in third instar larvae.

The data described in this paper, although demonstrating heterogeneity in ADH activity between wild caught male flies from different habitats, provide no evidence that ADH level *per se* in adults is an adaptive phenotype of the *Adh* locus. Middleton and Kacser (1983) have shown that *in vitro* measurements of ADH are poor predictors of the *in vivo* physiological consequences of enzyme variation. They conclude that *in vitro* differences in ADH activity between Adh^F/Adh^F and Adh^S/Adh^S homozygotes are unlikely to contribute to fitness differences between genotypes, at least in so far as ethanol metabolism is concerned.

Our observations support their conclusions by showing that the *Adh* polymorphism is unperturbed by environmental heterogeneity in ethanol levels. However, *D. melanogaster* populations do adapt to habitats with different levels of ethanol by some mechanism of tolerance, apparently unrelated to the variation in ADH activity.

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