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Relation between tolerance to ethanol and alcohol dehydrogenase (ADH) activity in *Drosophila melanogaster*: Selection, genotype and sex effects

M. Barbancho,
F. J. S. Sánchez-Cañete,
G. Dorado and
M. Pineda†

Departamento de Genética, Facultad de Ciencias, Universidad de Córdoba, 14071 Córdoba, Spain.
† Departamento de Bioquímica, Facultad de Ciencias, Universidad de Córdoba, 14071 Córdoba, Spain.

The suggestion of Oakeshott *et al.* (1984) that selection at the *Adh* locus, as a response to ethanol, is restricted to *D. melanogaster* laboratory-adapted populations, is tested in this paper with the "Lagar de los Reyes" (LR) lines. For this purpose, homozygous lines for the Adh^F and the Adh^S alleles were maintained on food supplemented with ethanol. After the selection, the ethanol tolerance and the ADH activity of the selected flies (LRSeF and LRSeS) were determined and compared with those of the control flies (LRCF and LRCS), maintained on standard medium. Then, the effects of the selection, genotype and sex, and the relation between ethanol tolerance and ADH activity were analysed. Our results fail to show a consistent correlation between ethanol tolerance and ADH activity in the adults of LR lines. Our findings also indicate that adaptation of *D. melanogaster* to ethanol-containing food could be accomplished without significant changes on the ADH activity in the adults. The possibility that the adaptation of *D. melanogaster* to environmental ethanol could be independent of the *Adh* locus is discussed.

INTRODUCTION

Since Lewontin and Hubby (1966) and Harris (1966) estimated by electrophoresis the extent of enzyme polymorphisms in populations of *Drosophila pseudoobscura* and humans, respectively, a considerable amount of genetic variation has been demonstrated both in plants and in animals (Powell, 1975; Nevo, 1978; Brown, 1979). However, the precise biological significance of the greater part of such variation is unknown to date. Due to the fact that it is difficult to determine whether selection is acting or not in nature, one of the most useful systems for studying the selective forces acting on a determined enzyme polymorphism is to analyse the effects of the selection and the genotype on a trait for which the allozyme variants differ in their biochemical properties (Lewontin, 1974; Clarke, 1975; Gibson *et al.*, 1979). For this reason, most of the studies done with *D. melanogaster* involve the alcohol dehydrogenase (ADH) allozymes and the alcohol tolerance.

Several evidences suggest that environmental ethanol is the main selective factor acting at the *Adh* locus:

(a) *D. melanogaster* ADH isozymes seem to play an important role in the detoxification of environmental ethanol. The ADH catalyzes the oxidation of ethanol to acetaldehyde, which is further transformed into acetate, the last oxidation being presumably also catalysed by ADH, as well as other enzymes (Clarke, 1975; David *et al.*, 1976; David, 1977; Deltombe-Lietaert *et al.*, 1979; Heinstra *et al.*, 1983).

(b) Natural populations of *D. melanogaster* are generally polymorphic for electrophoretic variants of ADH: ADH-F and ADH-S (Ursprung and Leone, 1965; Ward and Hebert, 1972; Pipkin *et al.*, 1976), Adh^F allele persisting in some cases at higher frequencies in habitats where ethanol is present at higher concentrations (Briscoe *et al.*, 1975; Hickey and McLean, 1980).

(c) *In vitro*, the FF homozygous strains show higher ADH activity than the SS homozygotes (Gibson, 1970; Ward and Hebert, 1972; Vigue and Johnson, 1973; Day *et al.*, 1974; Ward,

1974, 1975; Oakeshott, 1976*a*, *b*; Kamping and van Delden, 1978; McDonald and Ayala, 1978; Birley *et al.*, 1981; Sampsell and Steward, 1983; Kerver and van Delden, 1985). Additionally, the homozygous FF individuals usually have higher fitness on ethanol supplemented media than the SS ones (Gibson and Miklovich, 1971; Libion-Mannaert *et al.*, 1976; Oakeshott, 1976*a*; Thompson and Kaiser, 1977; Kamping and van Delden, 1978; van Delden and Kamping, 1983; Dorado and Barbancho, 1984).

(d) Increases of the Adh^F frequencies, the high-activity allele, have been observed when polymorphic populations are grown in ethanol supplemented media (Gibson, 1970; Bijlsma-Meeles and van Delden, 1974; van Delden *et al.*, 1975, 1978; Cavener and Clegg, 1978, 1981). In relation with this, McDonald *et al.* (1977) found higher ADH activities and greater amounts of ADH protein in adult flies which had been selected for increased tolerance to ethanol (David and Bocquet, 1977). These selected flies were fixed for the Adh^F allele, although the initial F and S frequencies were unknown.

However, some other evidence makes the selective effects of ethanol on the *Adh* locus ambiguous; each one of the above points can be questioned:

(a′) When assayed *in vitro*, the ADH allozymes are much more active on secondary than on primary alcohols (Vigue and Johnson, 1973; Day *et al.*, 1974; Morgan, 1975; Oakeshott, 1976*b*; Chambers *et al.*, 1978). Nevertheless, secondary alcohols are more toxic than their corresponding primary ones (David *et al.*, 1976, 1981) and secondary alcohols can not be used as energy sources (van Herewege *et al.*, 1980; Sánchez-Cañete *et al.*, 1986).

(b′) The Adh^F frequencies found in samples from inside Australian and Californian wineries were the same as in samples from outside the wine cellars, although the inside samples from Australian populations were more tolerant to ethanol than the outside ones (McKenzie and Parsons, 1974; McKenzie and McKechnie, 1978; Marks *et al.*, 1980).

(c′) Although the homozygous FF individuals exhibit both a higher ADH activity and a higher fitness on ethanol media than the homozygous SS, the heterozygotes FS show an intermediate ADH activity but their ethanol tolerance is either similar to the homozygous FF (Briscoe *et al.*, 1975), or higher than both homozygous FF and SS (Oakeshott, 1976*a*).

(d′) Increase in ethanol tolerance is not invariably associated with an increase in Adh^F frequencies or with an increased ADH activity (Gibson *et al.*, 1979; Oakeshott, 1979; Ziolo and Parsons, 1982; Oakeshott *et al.*, 1983, 1984).

Van Delden (1982) concluded that more direct detection of selection at the *Adh* locus should be obtained from the relation of the activities of the phenotypes "*in vitro*" to their survival in ethanol-supplemented media.

Oakeshott *et al.* (1984) suggested that the selection at the *Adh* locus as a response to ethanol is restricted to laboratory-adapted populations. The *Adh* locus in long-established laboratory populations might respond to ethanol selection because the background genotype evolved in natural populations had been changed.

The generality of this hypothesis has been tested in this paper, by investigating the responses to ethanol using long-established laboratory populations of *D. melanogaster* (LR lines). For this purpose, strains homozygous for the Adh^F and the Adh^S alleles were grown for many generations on ethanol supplemented food. The response to ethanol of the control (LRC) and selected lines (LRSe) has been studied by determining both ADH actvity and adult ethanol tolerance.

MATERIALS AND METHODS

Strains and culture conditions

Two strains of *Drosophila melanogaster*, one homozygous for the Adh^F allele (FF) and the other homozygous for the Adh^S allele (SS) were used. Each strain was obtained by intercrossing 15 homozygous stocks of the appropriate genotype. The stocks came from a sample collected in the "Lagar de los Reyes", winery in Baena, Córdoba (Spain). The two homozygous strains were maintained on standard medium (control lines; LRCF and LRCS) or on a medium containing 11 per cent ethanol (selected lines; LRSeF and LRSeS) for about 60 generations. More details on the lines and culture conditions are described elsewhere (Dorado and Barbancho, 1984).

Adult mortality measurements

In order to test adult tolerance to ethanol, samples from LRC and LRSe lines were cultured for one

generation on regular food. Then, 3- to 4-day-old flies from these cultures were fed one day more on fresh regular food. Adult mortality of these flies was then determined daily in hermetically closed vials containing 3 ml of different ethanol solutions (0, 1·25, 2·5, 5, 7·5, 10, 12·5 and 15 per cent, v/v). Each vial contained 10 males and 10 females, and 3 replicates for each concentration were made. The tests were carried out at approximately 25°C. For more details see Sánchez-Cañete *et al.* (1986).

ADH activity assays

The flies used for the ADH activity assays were derived from LRC and LRSe lines cultured in the way described above.

Samples of approximately 20 mg of adult flies were homogenised in 1 ml of cold 0·05 M tris-HCl buffer pH 8·6 for each line, genotype and sex. The weight and number of flies for each sample was recorded for further calculations. The homogenates were immediately centrifuged at 10,000 *g* for 10 min at 4°C and the resulting supernatants used as the enzyme source.

ADH activity was measured by following the reduction of NAD^+ to NADH at 340 nm with isopropanol or ethanol as substrates. The reaction mixture contained: 0·8 ml of 0·05 M tris-HCl buffer pH 8·6, 0·6 ml of 4mM NAD^+ in the same buffer, 0·1 ml of isopropanol or ethanol, and the appropriate amount (10–20 μl) of the enzyme extract. The reaction was started by addition of the enzyme extract, and the lineal NAD^+ reduction was registered during at least 3 minutes. All assays were performed at 30°C in a Beckman 24 double-beam recording spectrophotometer. One unit of ADH activity is defined as the amount of enzyme which reduces 1 μmol of NAD^+ per minute under assay conditions.

Five extracts were tested for each line, genotype and sex, and each one was assayed twice with isopropanol and twice with ethanol as substrates.

Protein in the extract was measured according to Bradford (1976), using bovine serum albumin as standard.

RESULTS

Ethanol tolerance

Fig. 1 shows adult ethanol tolerance, after 3 days of exposure, expressed as median lethal doses (LD_{50}), and table 1 represents a factorial ANOVA to test the extent of the effects of selection,

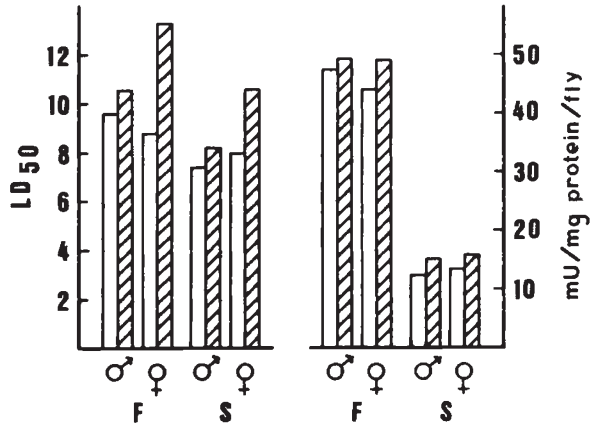

Figure 1 Median lethal doses (LD_{50}) to ethanol at third day, and ADH activities on isopropanol as substrate for each line, genotype and sex. Open boxes: LRC; dashed boxes: LRSe.

genotype and sex on ethanol tolerance. Significant differences can be observed between control (LRC) and selected (LRSe) lines (selection effect), FF and SS genotypes (genotype effect) and female and male individuals (sex effect).

Selection effect, quantified by the differences between LD_{50} from LRSe and LRC flies, and genotype effect, quantified by differences between LD_{50} from FF and SS flies are shown in table 2. In each case, statistical differences were analysed by means of a Tukey's test, using the error mean squares of the previous analysis of variance. FF and SS selected females reveal significantly higher LD_{50} values than their respective control ones, the selected males having a similar ethanol tolerance to the controls. So, the ethanol selection seems to have improved the tolerance to ethanol only of the females. Another observation from table 2 is that when females and males are compared, the females are more tolerant than the males only in the selected lines (LRSe), which again suggests that selection has improved the ethanol tolerance only of the females.

On the other hand, FF flies show significantly higher tolerance to ethanol than SS ones (with the exception of LRC females) (table 2).

ADH activity

To test if ADH activity is responsible for the improved ethanol tolerance in the females, it was assayed for each line, genotype and sex, using isopropanol and ethanol as substrates.

Table 3 shows relative ADH activities of each strain expressed in different units. Significant

Table 1 Factorial analysis of variance of the effects of lines, genotypes and sexes, on the LD_{50} to ethanol, and on the ADH activity. Enzyme activity is expressed as U/mg protein/fly.

Source of variation	LD_{50} ETOH			ADH Activity		
	df	Deviance	F	df	Deviance	F
Lines (L)	1	27·52	89·41**	1	0·01	0·11 ns
Sexes (S)	1	9·60	31·19**	1	1·14	9·98**
Genotype (G)	1	24·11	78·34**	1	111·02	970·05**
L×S	1	10·98	35·69**	1	0·08	0·74 ns
L×G	1	1·56	5·06*	1	0·15	1·30 ns
S×G	1	0·48	1·58 ns	1	0·01	0·07 ns
L×S×G	1	1·19	3·86 ns	1	0·06	0·54 ns
Residual	16	0·31		32	3·66	
Total	23			39		

* $P<0·05$; ** $P<0·01$; ns $P>0·05$.

differences are observed between females and males of the same genotype and line when ADH activity is expressed as U/mg protein. However, when ADH activity is expressed as U/fly, these differences disappear (data not shown). The latter fact seems to indicate that males and females have a similar level of ADH activity. Depending on the sex, a different number of flies was homogenised for each extract (26–28 males and 16–18 females), which corresponded to a similar weight (20± 0.5 mg/ml), so that ADH activities were definitively expressed as U/mg protein/fly (table 3 and fig. 1) and these units were used in further analysis.

As expected, isopropanol is a better substrate than ethanol (ADH activities being 4 to 6 times higher with the secondary than with the primary alcohol), and FF flies show 2–3 times higher ADH activities than SS ones (table 3).

A factorial ANOVA (summarised in table 1) was carried out to test the extent of the effects of selection, genotype and sex, on the ADH activity. Since high significant lineal correlation of ADH activities with isopropanol and with ethanol as substrates was observed ($r=0·99$; $P<0·001$), only ADH activities with isopropanol were used for the analysis.

Significant intergenotypic differences were detected, presumably as a result of the higher ADH activities in the FF flies. Significant differences were also observed between females and males. However, control and selected flies show similar ADH activities. Ethanol selection seems not to have increased the ADH activity of flies after 60 generations of selection (table 1).

In order to determine if the differences observed through the ANOVA, between lines, genotypes and sexes were consistent in each particular case, independent comparisons between pairs of strains (summarised in table 2) were carried out by means of a Tukey's test, using the error mean squares of the previous ANOVA. Again, significant differences in ADH activity were observed between FF and SS flies independently of sex and line. However, non significant differences were obtained either between lines and sexes at any of the pairs compared. Although the significant differences observed between sexes in the ANOVA (table 1) can be explained by the addition of non significant differences between females and males in each line and genotype, this is not the case of the differences between genotypes and lines. In fact, when the genotype effect is tested, FF extracts show significantly higher ADH

Table 2 Selection, genotype and sex effects on the LD_{50} to ethanol and on ADH activities with isopropanol as substrate. Statistical significance based on Tukey's test

Traits tested	Selection effect (LRSe vs. LRC)			Genotype effect (FF vs SS)			Sex effect (♀ vs. ♂)		
		♀	♂		LRC	LRSe		LRC	LRSe
LD_{50} ETOH	FF	4·45*	0·85 ns	♀	0·77 ns	2·67*	FF	−0·82 ns	2·78*
	SS	2·54*	0·72 ns	♂	2·22*	2·35*	SS	0·64 ns	2·46*
ADH Activity	FF	0·00 ns	−0·00 ns	♀	0·03*	0·03*	FF	0·00 ns	0·01 ns
	SS	0·00 ns	0·00 ns	♂	0·03*	0·03*	SS	0·00 ns	0·00 ns

* $P<0·05$; ns $P>0·05$.

Table 3 ADH activities with isopropanol (ISOH) and ethanol (ETOH) as substrates for each line, genotype and sex, expressed in different units. One unit of activity is defined as the amount of ADH that reduces one μmol of NAD^+ per minute

Line	Genotype and sex		U/mg protein		U/fly		U/mg protein/fly		
			ISOH	ETOH	ISOH	ETOH	ISOH	ETOH	I/E*
LRC	F	♂	1·209	0·200	0·0643	0·0106	0·0473	0·0078	6·05
		♀	0·824	0·127	0·0576	0·0089	0·0492	0·0076	6·48
	S	♂	0·343	0·078	0·0189	0·0043	0·0122	0·0028	4·41
		♀	0·258	0·063	0·0197	0·0048	0·0152	0·0037	4·11
LRSe	F	♂	1·197	0·192	0·0615	0·0099	0·0440	0·0071	6·22
		♀	0·795	0·129	0·0718	0·0116	0·0494	0·0080	6·17
	S	♂	0·368	0·079	0·0189	0·0041	0·0130	0·0028	4·66
		♀	0·289	0·070	0·0212	0·0052	0·0162	0·0040	4·09

* ISOH/ETOH.

activities than SS in all four comparisons, whereas when the selection effect is tested, none of the comparisons show significant differences between ADH activities (table 2).

Finally, it must be noted that females and males manifest similar ADH activities per individual although their weights are quite different. This should be taken in account when ADH activities are referred to fresh weight.

DISCUSSION

In order to facilitate the comparisons among strains when testing the effects of the selection, genotype and sex, the results have been summarised in table 4. The statistical signification of each comparison is based on the Tukey's test that had been previously carried out. In relation to genotype effect, FF flies of both control and selected lines show higher ADH activities than SS, and this difference is associated with a higher tolerance to ethanol in the males of both LRC and LRSe lines, but only to the females of the LRSe line. However, LRC FF females, although having a higher ADH activity than the SS, show a similar tolerance to ethanol. Thus, for the females, the ethanol tolerance-ADH activity relationship seems to have been modified by the selection. In other words, after the selection, FF females conserve higher levels of ADH activity than SS ones, but, in contrast to the controls, they have improved their tolerance to ethanol (genotype effect in table 4).

Moreover, the analysis of the selection effect (table 4) reveals that although both FF and SS females have increased their tolerance to ethanol as a response to the selection, this is not associated with an increase in ADH activity. All the above seems to imply that the selection must have acted on a different locus (loci) from the *Adh*. The same conclusion would be drawn if LRSe SS flies are compared with LRC FF ones (columns 9 and 10), since SS selected females show a higher LD_{50} and a lower ADH activity than FF controls, or when the effect of sex is tested (columns 11 and 12), since both FF and SS LRSe females are more tolerant but show similar ADH activities to their respective males. On the other hand, ADH activities and tolerance to ethanol are not correlated in LR lines, neither linearly ($r = 0{\cdot}599$ ns) nor by rank ($\tau = 0{\cdot}429$ ns).

Several authors have described in *D. melanogaster* positive relations between the tolerance to ethanol and the ADH activity (Oakeshott, 1976*a*; Thompson and Kaiser, 1977; van Delden *et al.*, 1978; Kamping and van Delden, 1978),

Table 4 Summary comparison of lines, genotypes and sexes for ethanol tolerance (LD_{50}) and ADH activities with isopropanol as substrate. Statistical differences are based on Tukey's test. The first value of each comparison is higher (+), no different (=), or lower (−) than the second one

	Genotype effect				Selection effect						Sex effect			
	FC vs. SC		FSe vs. SSe		FSe vs. FC		SSe vs. SC		SSe vs. FC		♀ F vs. ♂ F		♀ S vs. ♂ S	
Traits studied	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	LRC	LRSe	LRC	LRSe
Survival in ethanol (LD_{50})	=	+	+	+	+	=	+	=	+	−	=	+	=	+
ADH activity on isopropanol*	+	+	+	+	=	=	=	=	−	−	=	=	=	=

* U/mg protein/fly.

although in most of these papers the direct effect of the ADH activity on ethanol detoxification is questioned. Thompson and Kaiser (1977) pointed out that apparently the correlation between relative viability on ethanol-containing media and enzyme activity is not a direct correlation, while in the other resports the Adh^F allele was usually dominant over the Adh^S allele in terms of survival on ethanol (Oakeshott, 1976*a* even observed over-dominance of FS individuals), and co-dominant in terms of ADH activity. Recently Kerver and van Delden (1985) demonstrated that after 90 generations of ethanol selection an increase in tolerance was not accompanied by an increase in ADH activity in the adults, which is in agreement with our findings.

On the other hand, several multi-generation experiments have suggested that the Adh^F allele reaches high equilibrium frequencies in populations grown on ethanol containing media due to this higher ADH activity (Gibson, 1970; van Delden *et al.*, 1975, 1978; Cavener and Clegg, 1978, 1981; Vigue *et al.*, 1982). However, our results are in good agreement with those obtained by Gibson *et al.* (1979) and Oakeshott *et al.* (1983, 1984), which indicated that an increased tolerance to ethanol is not invariably associated with an increased frequency of the most active allele (Adh^F), or with an increased ADH activity. Oakeshott *et al.* (1984) have suggested that evidence of direct action of ethanol on the *Adh* locus seems to be restricted to "non-Australian populations which had spent three or more years in the laboratory prior to the experiment." However our LR lines came from a Spanish population and had been kept in the laboratory for approximately 4 years. Hence, Oakeshott and co-workers' suggestion that adaptation to environmental ethanol in *Drosophila melanogaster* can be independent of the ADH system, could be extended to non-Australasian long-established laboratory strains. Our selection experiments show that adaptation of *D. melanogaster* to ethanol-containing media can be carried out without significant changes in the constitutive levels of the *in vitro* ADH activities. Because the flies used to study the enzyme activity always came from the non ethanol-supplemented medium, the differences observed for ethanol tolerance between control and selected females could be due to the different induction of ADH activities caused by the ethanol present in the test tubes. Nevertheless, recent experiments carried out in our laboratory have not detected any induction of ADH activity by ethanol at concentrations used in selection (~10 per cent) in any of the lines, genotypes or sexes tested (paper in preparation). Kerver and van Delden (1985) even observed in males of selected strains that the presence of ethanol seems to arrest age-dependent increase in ADH activity, otherwise detected in regular food.

As expected, our ADH activity measurements in adults confirmed previous reports of a substantially higher *in vitro* activity of FF flies than of SS (Gibson, 1970; Day *et al.*, 1974; Oakeshott, 1976*a*, *b*; Kamping and van Delden, 1978; van Delden and Kamping, 1983; Kerver and van Delden, 1985), for both control and selected flies. The main role of ADH in the detoxification of alcohols in *D. melanogaster* is well established (Clarke, 1975; David *et al.*, 1976, 1981; van Delden *et al.*, 1978; Deltombe-Lietaert *et al.*, 1979; Dorado and Barbancho, 1984; Sánchez-Cañete *et al.*, 1986), but our results show no consistent correlation between adult ethanol tolerance and *in vitro* ADH activity. This conclusion is based on the facts (table 4) that: (a) F control females exhibit higher ADH activity but similar ethanol tolerance than S control females (column 1), (b) F and S selected females show no differences in ADH activity but have higher tolerance than their respective control females (columns 5 and 7), (c) S selected females present lower ADH activity and higher survival on ethanol than F control females (column 9), and (d) both F and S selected females manifest similar levels of ADH activity but higher tolerance to ethanol than their respective selected males (columns 12 and 14). Our findings are in agreement with those of Gibson *et al.* (1979), Ziolo and Parsons (1982), Oakeshott *et al.* (1983, 1984), and Kerver and van Delden (1985), who also found that an increase in ethanol tolerance is not consistently associated with an increase in ADH activity.

In conclusion, our results indicate that the adaptation of *Drosophila melanogaster* to ethanol-containing media could be accomplished without significant changes on the constitutive ADH levels in the adults. In addition to that, our data also indicate that this adaptation could be different for females and males. Different mechanisms must be involved in such a complex process. Amongst them, the ADH activity could play some role at juvenile life stages (Kerver and van Delden, 1985), but it is becoming more clear every day that other loci than the *Adh* locus must have an important role in the adaptation of *D. melanogaster* to ethanol-containing food.

As suggested Gibson *et al.* (1979), our results could also indicate that higher ADH activity might

be relevant in providing energy to flies that are tolerant to ethanol by some ADH activity-unrelated mechanism(s). In this way, the adaptation of *D. melanogaster* to environmental ethanol could be independent of the ADH system.

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