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Associations between Alcohol Tolerance and the Quantity of Alcohol Dehydrogenase in *Drosophila melanogaster* Isolated from a Winery Population

L. H. Schmitt,[A,B] *S. W. McKechnie,*[A] *and J. A. McKenzie*[C]

[A] Department of Genetics, Monash University, Clayton, Vic. 3168.
[B] Present address: Department of Anatomy and Human Biology, University of Western Australia, Nedlands, W.A. 6009.
[C] Department of Genetics, University of Melbourne, Parkville, Victoria 3052.

Abstract

Variation in alcohol dehydrogenase (ADH) and alcohol tolerance was investigated in Adh^F isofemale lines of *D. melanogaster* isolated from an Australian winery population. For three different tests of alcohol tolerance no association with levels of ADH activity was detected. However, among 156 lines a significant negative association occurred between larval ethanol tolerance and the quantity of ADH enzyme measured in adults. In addition, a positive relationship occurred between ADH quantity and adult survival in a sucrose–ethanol ingestion test carried out on 28 of the lines. No correlation of enzyme quantity occurred when adults were acutely exposed to ethanol vapour. These data indicate that ADH quantity and not ADH activity may be a more crucial physiological determinant of survival in high ethanol environments.

Introduction

One of the major and more difficult problems in population and evolutionary genetics is to elucidate the complex genetic basis of adaptive characteristics of animal populations (Tauber and Tauber 1978; Curtsinger and Laurie-Ahlberg 1982; Parsons 1982). The choice of characters which can be reasonably related to fitness, such as heavy metal tolerance in plants (Antonovics and Bradshaw 1970) and insecticide resistance in blowflies (McKenzie and Whitten 1984), will potentially reveal much about how new environments lead to changes in the genetic constitution of populations. One experimental system with considerable promise towards this end is tolerance to environmental alcohol by *Drosophila melanogaster*.

Substantial intraspecific genetic variation in ethanol tolerance occurs within this species (McKenzie and Parsons 1974; David and Bocquet 1975), which is commonly found in habitats containing ethanol. Laboratory experimentation leaves little doubt that under controlled conditions of exposure to ethanol, survival of *D. melanogaster* shows a dependence on genotype at the polymorphic *Adh* locus (Morgan 1975; Oakeshott *et al.* 1980; Daly and Clarke 1981; van Delden 1982). This relationship, however, has only been observed when the lines were inbred or had been established in the laboratory for long periods (Gibson and Oakeshott 1982). Although two studies of winery populations hint that the Adh^F allele is at higher frequencies in collections taken close to a rich alcohol source (Briscoe *et al.* 1975; Hickey and McLean 1980), this does not seem to be the case in all winery populations (Marks *et al.* 1980; Gibson *et al.* 1981), including that at Tahbilk in south-eastern Australia (McKenzie and McKechnie 1978). Recent findings indicate the possibility that adaptation to high environmental ethanol is more directly related to genes that regulate the quantity

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of ADH (McDonald *et al.* 1977). The existence of genetic variation at loci which affect ADH quantity or activity levels, loci other than *Adh* itself, is now firmly established (Birley *et al.* 1981). Some of these loci are tightly linked to the ADH structural gene, while others map to other parts of the genome (Thompson *et al.* 1977; McDonald and Ayala 1978; Laurie-Ahlberg *et al.* 1980; Maroni *et al.* 1982). Laboratory studies have indicated relationships between alcohol tolerance measures and both ADH activity (Kamping and van Delden 1978) and ADH quantity (Thompson and Kaiser 1977).

Natural variation in larval tolerance to ethanol occurs in the Tahbilk winery population. This variation has a polygenic basis (McKenzie and Parsons 1974; McKenzie and McKechnie 1978). Lines collected from the cellar or from the open vat fermentation area at vintage time are, on average, more tolerant than lines originating from the surrounding orchard area (McKenzie and Parsons 1974). Since there is a large background of genetic and biochemical information about the adaptation to alcohol by this species (see discussions and references in Anderson and McDonald 1981; Birley *et al.* 1981; van Delden 1982 and McKechnie and Geer 1984) this clinal variation provides a rare opportunity to study the genetical and biochemical structure of an adaptive polygenic character.

In the present study we investigate the contribution of ADH activity and ADH quantity variation to both larval and adult tolerance variation among a number of isofemale lines homozygous for the Adh^F allele. These lines were isolated from the Tahbilk winery over two vintage seasons.

Materials and Methods

Female flies were collected from the cellar, the fermentation area, from bottle traps set at the base of surrounding deciduous shade trees, and from the lemon orchard (all within 60 m of the cellar) during the vintage period (March to April) of 1979. Flies from the cellar and fermentation area were also collected during the 1980 vintage period. Lines were established for all homozygous Adh^F females that yielded only homozygous progeny (22 progeny were tested for each female). In all, 96 Adh^F lines were obtained in 1979 and 60 in 1980, and 11 lines that were found to contain the $Adh^{FCh.D.}$ gene using the method of Wilks *et al.* (1980) were discarded. There was no evidence for the presence of *Adh* null alleles. The larval tolerance test was on laboratory second-generation progeny of wild-caught females. Alcohol tolerance was determined at 20°C by placing 25 newly hatched larvae on standard semolina–treacle medium (without live or dead yeast) containing 9% (v/v) ethanol and scoring the number of eclosed adults (McKenzie and Parsons 1974). From all lines adult siblings of the tolerance-tested larvae were raised on the standard medium, with no alcohol or yeast. Adult male siblings 10–12 days old were weighed (approximately 30 per line), ground in 9 mM Tris–borate buffer containing 20 mM, EDTA, pH 8·7 (one fly per 10 μl), centrifuged at 10 000 *g* and the supernatant frozen in aliquots at −20°C. Using freshly thawed extract, ADH activity was measured according to Vigue and Johnson (1973) with ethanol and propan-2-ol (in separate assays) as substrates. Total protein was estimated (Lowry *et al.* 1951) using bovine serum albumin as a standard.

The number of ADH molecules relative to an arbitrary standard was estimated, on aliquots of the same extracts as activity, using the technique of radial immunodiffusion as modified from Lewis and Gibson (1978). Antibody to ADH was prepared against ADH extracted from many standard laboratory stocks including both Adh^F and Adh^S genes. A 20-ml mixture of 2·5% (w/v) agarose in the grinding buffer and 2·5% (v/v) anti-sera was poured into a 100-mm square Petri dish. Thirty-six wells, approximately 2·5 mm in diameter, were then punched in a grid pattern into the agar. As a standard for all ADH quantity determinations, several lines of *D. melanogaster*, including both Adh^F and Adh^S, were homogenized together, centrifuged and frozen in aliquots. A 6·5-μl sample of this standard was placed in each of six randomly chosen wells on every plate. A 1 in 8 dilution of the standard was placed in another six wells. Then for each of four lines to be tested against the standard on the same plate, 6·5-μl samples were placed in each of three wells and 6·5 μl of a 1 in 8 dilution placed in another three wells. Plates were kept at 4°C for approximately 18 h and then stained for ADH activity. Circle diameters

were measured and analysed by the parallel-slope assay method (Finney 1963), the square of the diameter estimating the log of the amount of ADH protein. For each plate, parallelism of the five lines (one standard and four unknowns) was tested. If the lines differed significantly from parallelism the analysis was repeated, after removing the aberrant line(s) which were then tested again at different concentrations. The quantity of ADH is expressed in RID units where one RID unit is the quantity in 6·5 μl of standard extract.

Of the lines collected in 1980, 28 were tested for ethanol tolerance in adults five laboratory generations after the test of larval tolerance. This test was modified from experiment 2 of Oakeshott *et al.* (1980). Up to five groups of about 20 adults were exposed for 24 h to a mixture of sugar and 15% (v/v) aqueous solution of ethanol in a sealed vial at 20°C. In a second test adults of 47 lines collected in 1980 were tested for acute exposure to ethanol vapour (experiment 3 of Oakeshott *et al.* 1980). This latter test measures survival after exposure to a high concentration of ethanol fumes for 45 min.

Results

Considering first the variation in ADH quantity (RID value) among all 156 lines, there were highly significant correlations between this and both average weight and total protein (Table 1; Fig. 1). A regression of the logarithm of ADH quantity on the logarithm of average weight (i.e. the power curve $y = ax^b$) gave a value for b equal to 1·04. If total protein was used instead of weight, the value for b was 1·05.

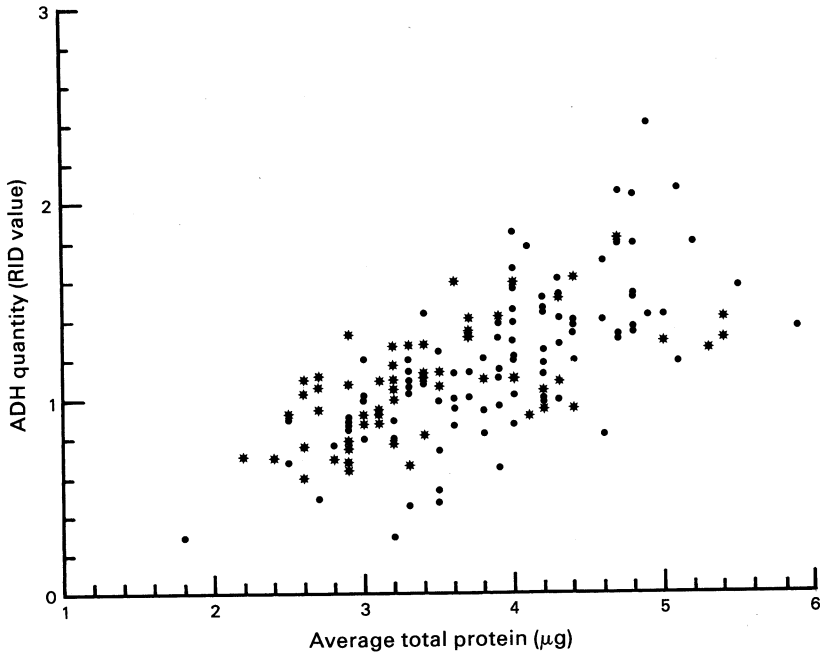

Fig. 1. Scatter diagram showing variation among Adh^F lines for average total protein and ADH quantity. ● Lines collected in 1979. ★ Lines collected in 1980.

Similar results were obtained for both years. These analyses suggest a simple linear relationship between ADH quantity and body size. Total protein was used as a correcting factor for variation in body size to estimate both specific ADH quantity and specific ADH activity. ADH quantity ÷ total protein (specific quantity) showed a highly significant positive relationship to specific ADH activity measured on both ethanol and propan-2-ol (Table 1).

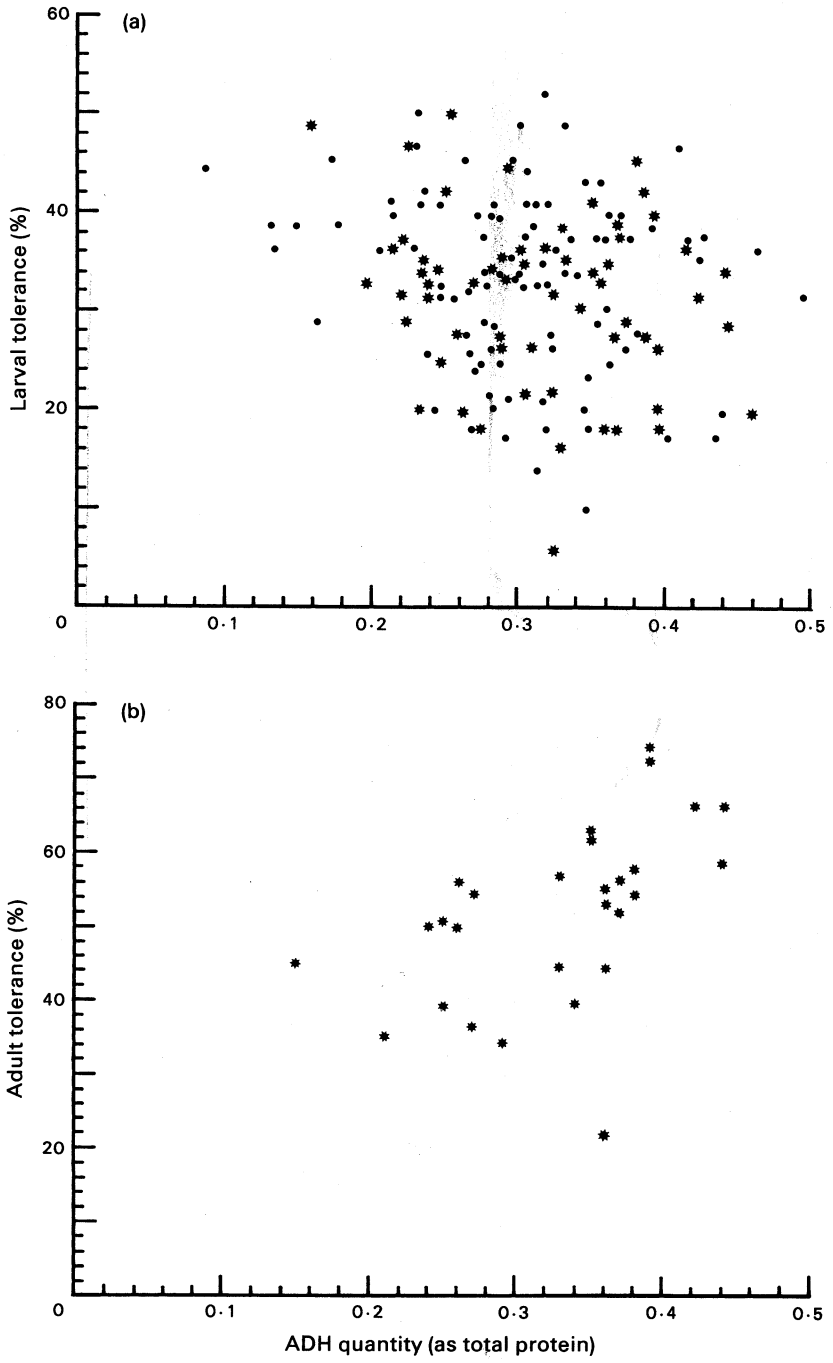

Fig. 2. Scatter diagram showing variation among lines for ADH quantity per total protein (specific ADH quantity) versus (*a*) larval alcohol tolerance, and (*b*) adult tolerance in the sucrose–ethanol ingestion test. Symbols as for Fig. 1.

The mean larval survival on 9% (v/v) ethanol-supplemented medium was 29·7%. Larval alcohol tolerance was not significantly correlated with body weight, protein content, or specific ADH activity measured using either ethanol or propan-2-ol as substrate (all analyses used the angular transformation of percentage tolerance). However, tolerance was significantly correlated (negatively) with the specific quantity of ADH (Table 1; Fig. 2). Multiple regression of alcohol tolerance on specific quantity and year of collection showed no statistically significant difference between years. This relationship between larval alcohol tolerance and specific ADH quantity was almost identical for the two years (Table 1). Since the tolerance cline is still apparent at Tahbilk, individual analyses of the data according to their collection site were carried out. The regression slopes for cellar–1979,–1980, fermentation area–1979,–1980 and outside–1979 were −10·2 (n = 35), −29·9 (39), −29·0 (32), −19·3 (21) and −50·8 (29), respectively. Multiple regression indicated these slopes to be homogenous and each insignificantly different from zero. Differences in tolerance between sites did not contribute to the overall association between specific ADH quantity and larval tolerance (i.e. there was no between-area effect).

Table 1. Regression slopes, correlation coefficients and probabilities for the larval tolerance test
*P = 0·05; **P < 0·01; ***P < 0·001; n.s., not significant

Dependent variable	Independent variable	Regression slope (correlation coefficient) 1979 (n = 96)	1980 (n = 60)	1979 + 1980
ADH quantity (RID value)	Weight (mg/individual)	1·68*** (0·57)	1·38*** (0·68)	1·56*** (0·59)
	Total protein (μg/ml)	0·37*** (0·68)	0·23*** (0·62)	0·30*** (0·66)
Specific activity, ethanol[A]	Specific ADH quantity (RID/μg)	9·83*** (0·57)	14·2*** (0·56)	12·4*** (0·52)
Specific activity, propan-2-ol[A]		63·6*** (0·66)	50·6*** (0·63)	57·4*** (0·63)
Tolerance (% survival)	Weight	−6·69 n.s. (−0·09)	−6·06 n.s. (−0·01)	−6·22 n.s. (−0·09)
	Total protein	−0·81 n.s. (−0·06)	0·17 n.s. (0·01)	0·09 n.s. (0·01)
	Specific activity, ethanol	−0·39 n.s. (−0·05)	−0·52 n.s. (−0·10)	−0·64 n.s. (−0·12)
	Specific activity, propan-2-ol	−0·18 n.s. (−0·13)	−0·31 n.s. (−0·19)	−0·19 n.s. (−0·13)
	Specific ADH quantity	−26·1* (−0·20)	−27·0 n.s. (−0·21)	−27·5** (−0·21)

[A] Expressed as optical density per minute per microgram.

Adult survival was determined for 28 lines tested by a one-day exposure to a solution of ethanol and sucrose. In this test, again, tolerance was not associated with ADH activity but showed a significant correlation with specific ADH quantity (r = 0·54, regression slope 86·9, P < 0·01). Here the association was positive in direction (Fig. 2). Regression analysis indicated a between-area component. Whereas a highly

significant association occurred for the 15 lines collected from the fermentation area (regression slope 113·4, $P < 0·01$), no significant association occurred for the 13 cellar lines (regression slope 28·1). Mean adult tolerances for these lines from the cellar ($\bar{x} = 47·7$) and fermentation area ($\bar{x} = 55·4$) were not significantly different. Tolerance in this test was significantly correlated with one other measured variable, average body weight ($r = 0·40$, $P < 0·05$).

Mean adult survival in the acute test in the desiccator was not significantly related to specific activity (as measured on ethanol or propan-2-ol), specific ADH quantity, nor to any other independently measured variable. However, survival in the acute and the ethanol–sucrose adult tolerance tests were correlated ($r = 0·40$, $P < 0·05$).

Discussion

Specific ADH activity measures, using both ethanol and propan-2-ol as substrates, were not related to survival in any of the tolerance tests. When specific ADH activity on ethanol and propan-2-ol were included in a multiple regression of larval tolerance there was no significant increase in the proportion of variation explained. These data indicate that ADH activity *per se* is not a crucial determinant of survival in any of the three tests. However, ADH quantity measurements have shown significant associations with tolerance.

About 4% of the variation in larval tolerance can be attributed to variation in the quantity of ADH among the lines. Conspicuous in this relationship is its negative direction – the lines with higher specific ADH quantities tended to have lower larval-to-adult survival. Given a number of reports, including this study, of high positive correlations between ADH activity and ADH quantity within electromorphs (McDonald *et al.* 1977; Thompson and Kaiser 1977; Lewis and Gibson 1978) this finding is surprising because high survival in ethanol tolerance tests is usually positively associated with high ADH activity (McDonald *et al.* 1977; Thompson and Kaiser 1977; Kamping and van Delden 1978). Thus, it has generally been postulated that high ADH activity results in more efficient detoxification of ethanol and hence high tolerance. What is now clear, however, is that this generalization does not always hold (Gibson and Oakeshott 1982). Under certain types of ethanol-tolerance tests, where adults are exposed to ethanol concentrations for short periods (Oakeshott *et al.* 1980; Shadraven and McDonald 1982), higher ADH activity relates negatively to increased survival. In this study the initial exposure of newly hatched larvae to 9% ethanol may have been analogous to these latter tests. Such results have been previously discussed in terms of a rapid build up of acetaldehyde, which is toxic in high concentrations (Gelfand and McDonald 1983). However, the concentrations of a number of other metabolites and tissue components are altered under an ethanol diet (Geer *et al.* 1983; McKechnie and Geer 1984) and at extreme levels any of these metabolites may result in larval death.

In the adult ethanol–sucrose tolerance test a higher level of association occurred between tolerance and ADH quantity. This association was positive and occurred in spite of a five-generation gap between ADH quantity determination and tolerance testing. The association occurred only among strains derived from females collected over the vats where fermentation fumes occur in excess. Perhaps these females do not represent a random sample of the adult tolerance variation over the whole Tahbilk area.

Previous studies have indicated a genetic basis for larval tolerance variation among isofemale lines isolated from the Tahbilk winery (McKenzie and Parsons 1974; McKenzie and McKechnie 1978). In addition a number of other studies (Birley *et al.* 1981; Maroni and Laurie-Ahlberg 1983) have indicated that populations of *D. melanogaster*, including the Tahbilk population (Birley 1984), harbour considerable variation in genes which determine ADH quantity. Therefore at least some of the variation among lines observed here, in both larval tolerance and adult ADH quantity, is likely to be genetically determined. The significant adult association between quantity and tolerance measured five generations later implicates a genetic component although we cannot exclude the possibility that environmental factors unique to individual lines, and propagated with the lines over generations, may have contributed to this correlation. The data from this study in themselves do not provide evidence for the genetic basis of the variation, since the biochemical measures and tolerance tests have not been replicated across generations on bottles. It is not possible to partition quantitatively the variation into that due to genetic effects, to culture bottles, or to experimental error. To minimize culture variation we took precautions to hold constant factors previously indicated to effect ADH quantity or alcohol tolerance or both: no yeast, no added alcohol (Bijlsma-Meeles 1979; Clarke *et al.* 1979).

A difficulty with interpretation arises because larval tolerance has been compared to ADH quantity measured in adults. However, if adult quantity levels are predictors of larval levels, as has been indicated for other lines [Maroni *et al.* (1982) found significant correlations of $r = 0{\cdot}87$, $r = 0{\cdot}96$ and $r = 0{\cdot}62$ for larval versus adult ADH activity in various sets of chromosome lines] an association between larval tolerance and larval ADH quantity is possible. This should be tested. ADH is of prime importance in ethanol metabolism—its quantity (Thomson and Kaiser 1977) and activity (Kamping and van Delden 1978) have previously been related to alcohol tolerance. The significant correlations here support the hypothesis of a direct physiological relationship between ADH quantity variation, whether it be genetically determined or not, and variation in these two measures of tolerance.

The absence of any obvious association between *Adh* gene frequency and larval tolerance variation in and around the Tahbilk winery (McKenzie and McKechnie 1978) lead us to consider only the Adh^F homozygotes. This was done to minimize variation in ADH activity and quantity due to *Adh* structural gene variability. As mentioned, natural populations including Tahbilk harbour considerable variation at sites which influence the quantity of ADH enzyme. Our results suggest that such sites affect variation in larval and adult alcohol tolerance. Some of these sites may be partly responsible for the gradient in larval ethanol tolerance in the winery population. Perhaps some elements will map close to the ADH gene because considerable natural variation among F alleles occurs in the *Adh* promoter region (Krietman 1983), from where ADH quantity could potentially be controlled (Maroni and Laurie-Alhberg 1983). Alternatively there may be unlinked ADH modifier genes (Birley *et al.* 1981; Maroni and Laurie-Ahlberg 1983). It would be interesting to know if any of the modifier elements described by these authors influence tolerance. In any event, if the variation in ADH quantity observed at Tahbilk is a reflection of underlying genetic variation at loci which regulate ADH, there is the potential in this field population to study the mechanisms by which selection moulds an adaptive character and to assess the importance of regulatory loci in the microadaptation of a natural population.

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