Regulatory differences in developmental expression of alcohol dehydrogenase are related to interspecies differences in ethanol tolerance of *Drosophila*

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Biochemical properties and expression patterns of alcohol dehydrogenase were compared among members of the *virilis* phylad of *Drosophila*. Quantitative differences in stage- and tissue-specific expression of ADH of *D. virilis*, *D. novamexicana*, *D. texana* and *D. americana* were observed. These differences seem to correlate with the temporal differences in ethanol tolerance among these species. Quantitative differences in ADH protein levels account for most, if not all, of the activity differences between species. Analysis of the interspecific hybrids revealed that these differences arise by a mechanism affecting monomer synthesis. A comparison with earlier studies, where independently evolved regulatory differences were related to adaptations, revealed contrasting underlying mechanisms.

Keywords: adaptation, alcohol dehydrogenase, developmental expression, *Drosophila*, ethanol tolerance, regulatory difference.

Introduction

The existence of variation in protein coding genes (structural) and in genes (regulatory) that control their expression patterns in natural populations is evidence of past mutational events. There has been a continued effort to understand the significance of such changes in genetic composition of natural populations in adaptive evolution (for reviews see Wilson *et al.*, 1977; MacDonald, 1983; Dickinson, 1991). While a great majority of such variations appears to be selectively neutral, evidence at the molecular level exists for the effects of positive Darwinian selection acting on both structural and regulatory genes to produce an adaptive phenotype (Crawford & Powers, 1989; Jessen *et al.*, 1991; Irwin *et al.*, 1992).

The demonstration of naturally occurring structural and regulatory variation at the *Adh* locus in populations of *Drosophila* and the ability of *Drosophila* alcohol dehydrogenase to detoxify and/or utilize environmental alcohol makes it especially amenable to studies of adaptation. Accordingly, several studies

relate intra- and interspecific differences in ADH activity levels to differential utilization of ethanol rich sources (McDonald & Avise, 1976). Consistent with this, several observations indicate that species breeding in fermenting fruits show higher ethanol tolerance than species breeding in fungi, decaying leaves and flowers (David & van Herrewege, 1983).

Among the *Drosophilids*, *D. melanogaster*, *D. lebanonensis* and *D. virilis* are the three species that exhibit the highest levels of ethanol tolerance in laboratory tests (David, 1988). Therefore, analysis of ADH in these species and their closest relatives provides an opportunity to investigate the structural and regulatory changes relative to a known adaptive function. Using this approach, Dickinson *et al.* (1984) and Laurie *et al.* (1990) demonstrated that the much higher level of tolerance to ethanol in adult *D. melanogaster* than *D. simulans* is due to a difference in ADH expression between the species.

In the *virilis* phylad of *Drosophila*, *D. virilis* is currently cosmopolitan (Spieth, 1979) and is the most abundant species found in the breweries of Japan (Choo & Oshima, 1974). Furthermore, populations of *D. virilis* exhibit latitudinal variation in ethanol tolerance patterns (David & Kitagawa, 1982); however, its closest relatives *D. novamexicana*, *D. texana* and *D.*

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americana show limited geographical distribution and are mainly North American populations (Throckmorton, 1975). In this report, a comparative analysis of the biochemical properties and expression of ADH throughout development at the levels of ADH activity and ADH protein concentration among members of the *virilis* phylad of *Drosophila* has been carried out to understand the basis for their differences in ethanol tolerance limits. We show important regulatory differences in developmental expression of ADH in *D. virilis* and its closely related species *D. novamexicana*, *D. texana* and *D. americana* and that the regulatory differences correlate with interspecies differences in ethanol tolerance patterns.

Materials and methods

Drosophila species

D. virilis (15010.1051.0), *D. novamexicana* (15010.1031.7), *D. texana* (15010.1041.22) and *D. americana* (15010.0951.0) were used in this study and were obtained from the National *Drosophila* Species Resource Center, Bowling Green, U.S.A. A wild-type stock of *D. virilis* was obtained from Banaras Hindu University, Varanasi, India. For comparative studies, two additional stocks of each species were also employed and have been described previously (Ranganayakulu, 1990; Ranganayakulu *et al.*, 1991). Our stocks of *Drosophila* were fixed for a slow allele of alcohol dehydrogenase (*Adh^s*) and were used for all experiments unless otherwise noted. The isolation of a *Adh^f* allele of *D. novamexicana* was described earlier (Ranganayakulu *et al.*, 1991) and was used in some of the interspecific crosses. Crosses between *D. virilis* and the rest of the species were carried out using three to four virgin females and two to three males. Flies were cultured on a standard corn meal agar medium at 25°C. For the production of hybrids, it was critical for the medium to be fresh. The flies did not mate on old medium nor did hybrid larvae survive.

Alcohol dehydrogenase activity

The larvae or pupae or adults of a given genotype were counted, weighed and homogenized in 0.02 M sodium phosphate buffer pH 7.3. The homogenate was centrifuged at 15,000 g for 20 min and the supernatant was used for alcohol dehydrogenase assay. For assaying ADH activity the spectrophotometric method of Schwartz *et al.* (1975) was used with ethanol as substrate. ADH units are expressed as nanomoles NAD⁺ reduced per minute. ADH activity in extracts of whole organisms or tissues is also detected by electrophoresis

followed by histochemical staining and quantified by densitometry (Ranganayakulu *et al.*, 1991).

Alcohol dehydrogenase purification

Thirty to forty grams of frozen adults of each species were used for the purification of ADH. The ADH purification protocol was similar to that of Sofer & Ursprung (1968) up to the point of resuspension of the pellet after 70 per cent ammonium sulphate precipitation in 10 mM sodium phosphate buffer pH 6.5. After resuspension it was dialysed extensively against the same buffer for 24 h. The dialysed protein was loaded on a Bio-Gel HTP column previously equilibrated with the same buffer. The protein was eluted with a linear gradient of 10–300 mM phosphate buffer pH 6.5. The protein was precipitated with 70 per cent ammonium sulphate and dialysed extensively with several changes of 50 mM sodium phosphate buffer. The dialysed protein was loaded on a cibacrome blue F₃GA column and the bound protein was eluted with freshly prepared 3 mM NAD. Fractions showing ADH activity were detected by a spot test employing the histochemical stain mixture. Those fractions showing substantial ADH activity were pooled and immediately dialysed against several changes of double distilled water overnight. The dialysed sample was freeze-dried and stored at –20°C.

SDS-PAGE

Polyacrylamide electrophoresis in the presence of SDS was performed using 15 per cent gel slabs as described by Laemmli (1970). After the electrophoresis the gels were washed with 7.5 per cent acetic acid for 30 min and stained with 0.1 per cent Coomassie blue R-250 in 50 per cent methanol and 75 per cent acetic acid for 4 h and destained with a solution containing 5 per cent methanol and 75 per cent acetic acid.

Molecular weight determination

The molecular weight of the ADH subunits was estimated by SDS-PAGE according to the procedure of Weber *et al.* (1972) using bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lactalbumin as molecular weight standards.

Protein estimation

Protein concentrations were determined using the Bio-rad protein assay reagents using bovine serum albumin as standard.

Enzyme kinetics

Michaelis-Menton constants for ethanol were determined for purified ADH from different species using the standard assay for ADH with varying concentrations of ethanol. The apparent K_m was estimated by a least squares regression fit.

Thermal stability of alcohol dehydrogenase

The pure enzyme solution was diluted to a concentration of $200 \mu\text{g ml}^{-1}$ with 0.02 M sodium phosphate buffer $\text{pH } 7.5$ and incubated in a water bath at 45°C . At intervals of 10 min aliquots were withdrawn and assayed using the standard assay procedure. Control enzyme assays were carried out with enzyme samples that were not subjected to heat treatment. The thermal stability of the enzyme was determined by measuring the loss of enzyme activity on heating compared with the control values. All assays were duplicated and two replicates of each sample were tested in each experiment.

Immunological methods

Production of polyclonal antisera. Polyclonal antibodies to purified *D. virilis* ADH were raised in New Zealand White rabbits. Briefly, the lyophilized ADH protein was dissolved in 50 mM sodium phosphate buffer $\text{pH } 7.0$ containing 0.9 per cent NaCl and $500 \mu\text{g}$ of such protein was emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites. After 4 weeks, two boosts at intervals of 1 week were given. Blood samples were collected after the final boost from the ear vein in centrifuge tubes. After allowing to stand at room temperature for 2–3 h and overnight at 4°C without disturbing, the blood clot was allowed to settle by centrifuging in a clinical centrifuge at maximum speed for 10 min. To the yellowish serum, 0.1 per cent Thimersol was added and stored at -20°C as $100 \mu\text{l}$ aliquots.

Estimation of cross-reacting material. ADH cross-reacting material was estimated by the radial immunodiffusion method of Mancini *et al.* (1965) using antiserum at a 1 per cent final concentration. Forty organisms of each developmental stage (actively feeding third instar larvae, late pupae (eyes and wing pads visible) or adults of a given age) were homogenized in 1 ml of 0.02 M sodium phosphate buffer $\text{pH } 7.3$, and spun at 7000 r.p.m. in a cooling microfuge for 10 min. Ten microlitres of supernatant were placed in 3 mm wells and allowed to diffuse for 48 h at 4°C and the

precipitin rings were visualized using the ADH histochemical stain. The relative CRM levels were directly read from a standard curve ($r=0.99$) obtained by plotting ring diameter against log concentration of antigen (Fahey & Mckelvey, 1965). *D. virilis* ADH was used as antigen for the standard curve. Furthermore, a single radial immunodiffusion experiment performed with a defined quantity of purified ADH from each species showed no significant variation in the ring diameter.

Ethanol tolerance tests

Adult fly cultures were subjected to ethanol treatment according to the method of David & Bocquet (1977). Larvae were tested for survival on ethanol supplemented media. Briefly, adult flies were allowed to lay eggs on agar plates and hatched first instar larvae were transferred to instant *Drosophila* medium (Carolina Biological) supplemented with ethanol. Cultures maintained without ethanol (but with 2 per cent sucrose or instant medium) served as control.

Results

Developmental programme

The developmental profiles for ADH activity in *D. virilis*, *D. novamexicana*, *D. americana* and *D. texana* are shown in Fig. 1. It is clear from the data that the activity increases during larval development until the third instar stage, then falls off rapidly during the pupal stage and begins to rise again at eclosion. Even though these species show developmental profiles of similar shape there are significant quantitative differences. Firstly, of the tested species *D. virilis* consistently has higher activity at the larval and adult stages of development. Secondly, between 2 and 4 days post-eclosion *D. virilis* shows a rapid increase of ADH activity over other species.

Although the differences in levels of ADH activity observed in these species using crude enzyme extracts are informative, a quantitative comparison of the ADH protein levels reflects the underlying gene activity. To this end polyclonal antibodies were raised against the purified *D. virilis* ADH. These antibodies were shown to produce single and continuous precipitin lines on cross-reaction with the antigen from the rest of the species (Ranganayakulu *et al.*, 1991). Utilizing this antiserum the ADH protein concentrations were determined by the single radial immunodiffusion method. From the results presented in Table 1, it is clear that *D. virilis* and *D. americana* show the highest amounts of ADH protein at the third instar larval stage. In all

Fig. 1 Developmental time course of ADH activity. Each point represents the mean of determinations made on four separate extracts. (□) *D. virilis*; ♦, *D. novamexicana*; ■, *D. texana* and ◇, *D. americana*. The eclosion times at 25 °C after oviposition for *D. virilis*, *D. novamexicana*, *D. texana* and *D. americana* are 13.5, 13.0, 13.0 and 15.5 days, respectively.

Table 1 Relative CRM levels at different developmental stages

Species	Developmental stage			
	III instar larvae	Pupae	Adults	
			2-day-old	5-day-old
<i>D. virilis</i>	0.28 (7.3 ± 0.23)	0.08 (4.5 ± 0.11)	0.89 (10.1 ± 0.29)	1.00 (10.5 ± 0.23)
<i>D. novamexicana</i>	0.14 (5.6 ± 0.14)	0.10 (5.0 ± 0.11)	0.08 (4.6 ± 0.18)	0.37 (7.8 ± 0.36)
<i>D. texana</i>	0.25 (6.9 ± 0.18)	0.09 (4.6 ± 0.13)	0.14 (5.6 ± 0.11)	0.30 (7.2 ± 0.17)
<i>D. americana</i>	0.27 (7.2 ± 0.15)	0.07 (4.3 ± 0.14)	0.17 (6.2 ± 0.15)	0.20 (6.7 ± 0.22)

All values are expressed relative to *D. virilis* adult CRM levels. Values in parentheses represent mean ring diameter and standard error of triplicate samples.

species pupae show lower levels of ADH protein than those of larvae. Furthermore, it was observed that the 2-day-old *D. virilis* adults show almost tenfold higher levels of ADH protein than that of pupae. On the other hand, the rest of the species show either equal or nearly twofold higher ADH protein over their respective pupal levels. There is a gradual increase in all species in their ADH protein level up to the fifth day stage but their level remains significantly lower than that of *D. virilis*. The CRM profiles corroborate the observed

differences in the developmental profiles of ADH activity. Thus it is clear that the fluctuations in the activity of ADH during development are reflected in the steady-state levels of ADH protein. These differences in steady-state levels of ADH protein found in the standard stocks used in preceding experiments are widespread in the respective species. We tested at least two additional stocks (all *Adh^s*) of each species and found no significant differences compared with the standard stocks (Ranganayakulu, 1990).

Fig. 2 Mortality on ethanol. (a) Larvae were scored as percentage survived to eclosion. (b) Adult mortality after 2 days of exposure to various concentrations of ethanol. $\square$, *D. virilis*; $\blacklozenge$, *D. novamexicana*; $\blacksquare$, *D. texana*; and $\bullet$, *D. americana*.

Ethanol tolerance

As ADH is involved in the detoxification of alcohols, the larvae and adults of these species were subjected to alcohol treatments of various concentrations to test whether there is any correlation in ADH activity levels and ethanol tolerance limits. From the results presented in Fig. 2 it is clear that among the tested species *D. virilis* has the highest tolerance. Furthermore, these differences are much more striking at the adult stage. Thus the species-specific differences in ADH activity levels during development may be significant in terms of adaptation of these species to alcohol environments.

Characterization of the ADH protein

As the activity differences between species represent differences in the steady-state level of the ADH protein, it is instructive to assess the structural relatedness of the protein from each species. As the first step, the enzyme from each species was purified and the purity of the samples was judged by SDS-PAGE. In all cases they were found to be greater than 90 per cent pure (data not shown). The pure enzyme solution was used to determine some biochemical features of ADH in different species. From the results presented in Table 2, it is clear that *D. americana* and *D. texana* show similar Michaelis constants and pH optima. Even though *D. virilis* and *D. novamexicana* have nearly identical K_m values, their pH optima values are different. Among these species, *D. virilis* is the only species with two pH optima values. Furthermore, the thermal stability of ADH from each species is shown in Fig. 3. The enzymes clearly differ in their stability patterns suggesting differences in the primary structure of the protein.

Table 2 Biochemical properties of ADH

Species	Subunit molecular weight	pH optima	Ethanol kinetic parameters†	
			$K_m(\text{app})$ (mM)	$V_{\max}(\text{app}) \times 10^3$ units/mg protein
<i>D. virilis</i>	27 800	8.5, 10.0	5.83 ± 0.19	5.07 ± 0.12
<i>D. novamexicana</i>	27 800	9.5	5.35 ± 0.32	2.50 ± 0.11
<i>D. texana</i>	27 800	9.0	7.28 ± 0.21	2.67 ± 0.10
<i>D. americana</i>	27 800	9.0	7.68 ± 0.32	2.51 ± 0.17

†Each value represents mean and standard error of three independent determinations and values are calculated according to Lineweaver & Burk (1934).

Tissue-specific expression of ADH

The fact that there are substantial quantitative differences both in ADH activity and CRM level between species raises the pertinent question as to whether these differences exist uniformly in all tissues. In order to define the expression of ADH, different tissues from individual organisms were dissected out from larvae as well as adults and their ADH levels were quantified by electrophoretic separation and densitometry. These results were corroborated by estimating the CRM levels of individual organs (Tables 3 and 4). Species-specific differences in larval tissues are clearly evident in that *D. virilis* shows higher activity in anterior midgut and fatbody whereas *D. americana* shows highest

Fig. 3 Thermal stability of purified alcohol dehydrogenase at 45°C. □ *D. virilis*, ♦ *D. novamexicana*, ■ *D. texana*, ◇ *D. americana*.

activity in midgut and carcass. On the other hand, at the adult stage *D. virilis* shows highest activity in all the tested tissues. When a comparison was made of CRM levels of three larval tissues and four adult tissues the patterns were found to be similar to those of gel scanning patterns.

Genetic basis of activity variation

The quantitative variation in CRM could result from differences in synthesis or turnover of the gene product resulting in an altered steady-state level. A distinction between these alternatives can be made by analysing the activity patterns in interspecific hybrids. From the results presented in Table 5 it is clear that activity patterns of interspecific hybrids are suggestive of additive inheritance but in *D. virilis* × *D. novamexicana* F₁ hybrids, the activity per organism is much lower than the midparent value. This appears to be a strain-specific phenomenon because in crosses with other stocks of *D. novamexicana* the F₁ hybrids show strict additive inheritance (Ranganayakulu, 1990). If activity patterns were to be determined by post-translational enzyme degradation under trans-acting control, inheritance patterns of a dominant recessive nature would be obtained (Ganschow & Schimke, 1969; Lai & Scandalios, 1980; King & McDonald, 1983). Thus these results suggest that the differences are likely to be arising out of differences in enzyme synthesis.

As the allozymes coded by *D. virilis* and *D. novamexicana* differ in electrophoretic mobility patterns a strategy designed by Dickinson (1980) was used to compare the relative expression of the alleles in interspecific hybrids. From the results shown in Fig. 4 it is clear that the allozyme characteristic of *D. virilis* is predominantly expressed. Furthermore, the subunit

Table 3 Relative ADH activity levels in different tissues of larvae and adults

Species	Larval tissue†				Adult tissue‡			
	Anterior midgut	Midgut	Fatbody	Carcass	Head	Midgut	Hindgut	Fatbody
<i>D. virilis</i>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
<i>D. novamexicana</i>	0.30 ± 0.10	0.80 ± 0.13	0.30 ± 0.10	1.00 ± 0.09	0.47 ± 0.08	0.19 ± 0.07	0.22 ± 0.07	0.41 ± 0.09
<i>D. texana</i>	0.13 ± 0.05	1.05 ± 0.15	0.64 ± 0.17	0.51 ± 0.08	0.52 ± 0.11	0.54 ± 0.12	0.25 ± 0.08	0.25 ± 0.07
<i>D. americana</i>	0.21 ± 0.06	1.99 ± 0.26	0.70 ± 0.12	1.00 ± 0.15	0.26 ± 0.08	0.29 ± 0.08	0.37 ± 0.10	0.13 ± 0.06

† Actively feeding third instar larvae were used as source of organs. In each case 15 anterior midguts, 8 midguts, 3 carcasses or fatbodies of three larvae were homogenized in 30 µl buffer, centrifuged at 1000 r.p.m. for 2 min at 4°C and 3 µl of the supernatant was used for activity assay or for the determination of CRM levels.

‡ 3–6-day-old adults were used as source of organs. In each case, 4 heads, 20 midguts, 20 hindguts or fatbodies (along with abdominal bodywall) of two adults were homogenized in 30 µl buffer and processed as above.

All values are expressed relative to *D. virilis* tissue activity levels.

Table 4 Relative ADH protein levels in different tissues of larvae and adults

Species	Larval tissue			Adult tissue			
	Anterior midgut	Midgut	Fatbody	Head	Midgut	Hindgut	Fatbody
<i>D. virilis</i>	1.00 (11.0 ± 0.47)	1.00 (11.1 ± 0.36)	1.00 (11.0 ± 0.31)	1.00 (9.5 ± 0.35)	1.00 (7.4 ± 0.21)	1.00 (7.0 ± 0.12)	1.00 (11.2 ± 0.54)
<i>D. novamexicana</i>	0.22 (7.7 ± 0.12)	0.86 (10.4 ± 0.18)	0.23 (7.6 ± 0.24)	0.53 (8.0 ± 0.23)	0.21 (4.5 ± 0.21)	0.27 (4.2 ± 0.12)	0.32 (9.0 ± 0.31)
<i>D. texana</i>	0.10 (6.0 ± 0.14)	1.13 (11.0 ± 0.20)	0.71 (9.1 ± 0.18)	0.45 (7.6 ± 0.24)	0.64 (6.5 ± 0.18)	0.27 (4.4 ± 0.08)	0.20 (8.0 ± 0.30)
<i>D. americana</i>	0.20 (7.6 ± 0.17)	2.10 (12.2 ± 0.23)	0.68 (10.1 ± 0.18)	0.19 (5.4 ± 0.21)	0.28 (4.7 ± 0.16)	0.36 (4.7 ± 0.07)	0.10 (6.6 ± 0.20)

Values in parentheses represent mean ring diameter and standard error of triplicate values.

For sample preparation see footnotes of Table 3. For each set of determinations *D. virilis* tissue ADH levels were used as standards (1.00) and all other values are relative.

Table 5 ADH activity levels of parents and hybrids

Species	Developmental stage			
	Larvae		Adults	
	Activity per organism	Specific activity	Activity per organism	Specific activity
Parents				
<i>D. virilis</i>	18.0 ± 3.0	92.0 ± 7.5	35.0 ± 5.0	157.0 ± 14.0
<i>D. novamexicana</i> (<i>Adh^s</i>)	10.0 ± 2.0	52.0 ± 5.0	13.0 ± 2.3	48.0 ± 5.7
<i>D. texana</i>	14.0 ± 1.6	56.0 ± 4.0	19.0 ± 2.3	57.0 ± 7.0
<i>D. americana</i>	16.0 ± 3.0	47.0 ± 5.7	16.0 ± 1.6	49.0 ± 5.0
F ₁ hybrids				
<i>D. virilis</i> × <i>D. novamexicana</i> (<i>Adh^s</i>)	15.0 ± 2.3 (14.0)	68.0 ± 6.5 (72.0)	18.0 ± 3.3 (24.0)	100.0 ± 9.3 (102.5)
<i>D. virilis</i> × <i>D. texana</i>	16.0 ± 1.6 (16.0)	78.0 ± 6.3 (74.0)	27.0 ± 5.3 (27.0)	104.0 ± 6.9 (107.0)
<i>D. virilis</i> × <i>D. americana</i>	17.0 ± 2.3 (17.0)	62.0 ± 4.6 (69.5)	20.0 ± 2.7 (25.5)	107.0 ± 11.0 (103.0)

Values in parentheses represent expected results from additive inheritance patterns and are predicted by the mean of the two parental values.

ratios observed in the hybrid are in good agreement with those predicted from the activity differences of the parents, suggesting that the activity differences are entirely consistent with a mechanism affecting monomer synthesis.

Discussion

The data presented in this report show differences in temporal patterns of ethanol tolerance among closely related species of the *virilis* group of *Drosophila*. These

differences seem to be related to the observed quantitative differences in ADH levels of these species. Similar conclusions have been reached for other *Drosophila* species (McKenzie & Parsons, 1972; McDonald & Avise, 1976; Dickinson *et al.*, 1984). To determine the regulatory versus structural basis of such interspecific differences in ADH levels, a comparative analysis of the biochemical properties and expression patterns of ADH has been carried out. The immunological data strongly suggest that most, if not all, of the activity variation between species is accounted for by

Fig. 4 Regression analysis of observed and predicted dimer proportions of allelic enzymes in hybrids of *D. virilis* × *D. novamexicana* (*Adh*^I). The x-axis represents the dimer proportions measured in hybrids by densitometry. The y-axis represents the dimer proportions predicted by the binomial theorem from relative activity ratios of the parents measured by electrophoresis of mixed samples. Bars represent standard errors of four determinations. The line drawn has a slope of 1.00 and represents the line on which the data would lie if the observed and predicted dimer proportions matched perfectly. ♦, III instar larvae; □, adults.

variation in the concentration of ADH protein. Furthermore, such differences were found to be stage- and tissue-specific. It is interesting to note that such differences in quantitative levels of ADH are more pronounced at the adult stage than at the larval stage. These differences seem to arise by a difference in relative rates of synthesis in these species. The additive inheritance of activity patterns and the observed good correlation of dimer ratios in interspecific hybrids are consistent with such a conclusion; however, a detailed analysis of the interspecific hybrids of *D. virilis*/*D. novamexicana* revealed subtle differences that specifically affect the heterodimer stability in the adult head and hindgut tissues (G. Ranganayakulu & A. R. Reddy, unpublished data).

From a biochemical characterization of the ADH protein, it is evident that the ADH present in these species has identical molecular weight but differences found in pH optima and kinetic parameters represent structural differences. Although it is difficult to assess the biological significance of these differences they at least indicate the existence of differences in the catalytic site of ADH among these species. David & Kitagawa (1982), however, attributed the high ethanol tolerance in *D. virilis* to a highly active ADH, based on

spectrophotometric and electrophoretic assays. Similarly, Juan & Gonzalez-Duarte (1981) found a closer similarity in ethanol kinetic parameters of ADH between *D. virilis* and *D. melanogaster* and interpreted it as an adaptation to the ecology of the species.

Our central conclusion from the present study is that there seems to be increased ethanol tolerance in *D. virilis* over its closely related species based largely, but not completely, on a change in gene regulation. In many ways this looks like a situation parallel to the comparison made earlier between *D. melanogaster* and *D. simulans* (Dickinson *et al.*, 1984) where the differences in *Adh* expression arise due to one or more *trans*-acting genes (Laurie *et al.*, 1990). In the present case simple genetic mechanisms all under *cis*-control seem to account for such differences. A similar analysis of ADH expression in *D. lebanonensis* and its closely related species should be interesting. The recruitment of lysozyme in the stomach of ruminants and colobine monkeys is another good example of convergent adaptive evolution (Stewart *et al.*, 1987). In this case, the large-scale production of lysozyme by the stomach lining involved gene duplication as well as a regulatory change in gene expression (Dobson *et al.*, 1984; Irwin *et al.*, 1989).

Among these species, the enzyme ADH is coded by homologous genes (Ranganayakulu *et al.*, 1991) and yet significant differences in biochemical properties and stage-specific expression of ADH were found. These differences are much more striking between *D. virilis* and the rest of the species. It is tempting to speculate that this is a clear case of adaptive evolution. Consistent with this are the observations that *D. virilis* is often collected from breweries and seems to be attracted by alcohol-rich environments (Choo & Oshima, 1974; Spieth, 1979); however, the natural habitat of these species seems to be the rotting bark of a limited number of deciduous tree species which is not likely to contain a high amount of alcohol (Throckmorton, 1975). This suggests that high ethanol tolerance in *D. virilis* may be a recently evolved mechanism for adaptation to alcohol-rich habitats. In general, ethanol as a selective agent can act either at the pre-adult or adult stages of *Drosophila*. It was shown earlier that in *D. melanogaster*, the adaptation to ethanol-rich media was mainly accomplished during the juvenile life stages and that the maternal ADH has a significant role in that process (Kerver & van Delden, 1985; Kerver & Rotman, 1987). In the *virilis* phylad of *Drosophila*, ADH is not maternally encoded and zygotic transcription of *Adh* is not seen until the 24th hour of embryogenesis (G. Ranganayakulu, unpublished data). Therefore, in these species, if ethanol tolerance is related more to adult exposure to

ethanol vapour during feeding and/or oviposition than to larval resources, one would expect to see more differences in patterns of expression of ADH at the adult stage than at the larval stage. The results presented in this report are consistent with such a conclusion.

Finally, a complete understanding of the significance of interspecific differences in ADH activity will depend on clarification of the processes by which they are established, including an adequate description of the intraspecific variants that might serve as raw material for evolution of fixed differences. In view of this, alcohol dehydrogenase expression was analysed in three stocks of each species of the *virilis* phylad of *Drosophila* and only in *D. virilis* were quantitative variants associated with tissue-specific differences in ADH expression identified (Ranganayakulu, 1990; Ranganayakulu *et al.*, 1991). Earlier studies, however, on chromosome substitution lines in *D. melanogaster* suggest that quantitative variation in enzyme activity is common and extensive between lines (Laurie-Ahlberg *et al.*, 1980, 1982). It follows then that a detailed analysis of ADH similar to the one in the present study employing a wide variety of stocks of each species of the *virilis* phylad of *Drosophila* is needed for an evaluation of the adaptive significance of such polymorphisms.

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