Inhibition of Alcohol Dehydrogenase After 2–Propanol Exposure in Different Geographic Races of *Drosophila mojavensis*: Lack of Evidence for Selection at the *Adh–2* Locus

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ABSTRACT High frequencies of the fast allele of alcohol dehydrogenase–2 ($Adh–2^F$) are found in populations of *Drosophila mojavensis* that inhabit the Baja California peninsula (race BII) whereas the slow allele ($Adh–2^S$) predominates at most other localities within the species' geographic range. Race BII flies utilize necrotic tissue of pitaya agria cactus (*Stenocereus gummosus*) which contains high levels of 2–propanol, whereas flies from most other localities utilize different cactus hosts in which 2–propanol levels are low. To test if 2–propanol acts as a selective force on *Adh–2* genotype, or whether some other yet undetermined genetic factor is responsible, mature males of *D. mojavensis* lines derived from the Grand Canyon (race A) and Santa Catalina Island (race C), each with individuals homozygous for $Adh–2^F$ and $Adh–2^S$, were exposed to 2–propanol for 24 h and ADH–2 specific activity was then determined on each genotype. Flies from five other localities homozygous for either the fast or slow allele also were examined. Results for all reported races of *D. mojavensis* were obtained. 2–propanol exposure inhibited ADH–2 specific activity in both genotypes from all localities, but inhibition was significantly less in two populations of race BII flies homozygous for $Adh–2^F$. When F/F and S/S genotypes in flies from the same locality were compared, both genotypes showed high 2–propanol inhibition that was not statistically different, indicating that the F/F genotype alone does not provide a benefit against the inhibitory effects of 2–propanol. ADH–1 activity in female ovaries was inhibited less by 2–propanol than ADH–2. These results do not support the hypothesis that 2–propanol acts as a selective factor favoring the $Adh–2^F$ allele. *J. Exp. Zool. (Mol. Dev. Evol.) 304B:000–000, 2005.*

INTRODUCTION

Drosophila mojavensis Patterson and Crow is a cactophilic drosophilid that inhabits a broad geographic range within the Sonoran Desert of northwestern Mexico, southern Arizona, and southeastern California (Heed, '78). Isolated populations of *D. mojavensis* also are found on Santa Catalina Island, off the southern California coast, and at the Grand Canyon in northwestern Arizona (Heed and Mangan, '86; Ruiz et al., '90). Populations of *D. mojavensis* have been subdivided into different races, or subspecies, based on a number of criteria, including morphological and genetic differences, and the species has become an important model organism for understanding the role of geographic isolation and genetic divergence during speciation (Mettler, '63; Zouros, '73; Ruiz et al., '90; Markow and Hocutt, '98). Within each geographic area populations of *D. mojavensis* generally utilize necrotic tissue (rots) of a specific local host cactus as feeding and breeding sites, although shifts in host cacti are seen among the different geographic areas (Table 1).

Race B of *D. mojavensis* from southern Arizona and northwestern Mexico was split into subraces BI (mainland) and BII (Baja California

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TABLE 1. Host cacti of the different geographic races of Drosophila mojavensis *in the southwestern U. S. and northwestern Mexico*

Race	Subspecies	Geographic region	Host cactus
A	*D. mojavensis mojavensis*	S. Calif. desert, Grand Canyon, Ariz.	barrel cactus (*Ferocactus cylindraceus*)
BI	*D. mojavensis sonora*	S. Ariz., NW Mexico mainland	organpipe cactus (*Stenocereus thurberi*)[1]
BII	*D. mojavensis baja*	Baja California peninsula	pitaya agria (*Stenocereus gummosus*)
C	*D. mojavensis wrigleyi*[2]	Santa Catalina Island	prickly-pear cactus (*Opuntia littoralis*)

[1]In a restricted coastal area of northwestern Sonora, Mexico, in the region of El Desemboque, pitaya agria is found and used by *D. mojavensis sonora* as a host.
[2]The population of *D. mojavensis* on Santa Catalina Island was originally grouped with race A based on similarities of chromosome inversions (Ruiz et al., '90), but more recent allozyme and microsatellite data indicate that this population is distinct (Hocutt, 2000; Ross CL and Markow TA, in preparation).

peninsula=Baja) (Table 1) based mainly on the large differences seen in allele frequencies at the alcohol dehydrogenase–2 (*Adh–2*) locus (Zouros, '73). Gene duplication in *D. mojavensis* has resulted in separate *Adh* loci, *Adh–1* and *Adh–2*, that code for two distinct and functional forms of alcohol dehydrogenase (ADH; EC 1.1.1.1) which show different charge characteristics, temperature sensitivities, and developmental expression (Batterham et al., '83a, b; Atkinson et al., '88). *Adh–1* is expressed in eggs and larvae, as well as ovaries of adult females, and has been shown to be monomorphic (Heed, '78; Matzkin and Eanes, 2003). The *Adh–2* locus is expressed in adults of both sexes. The well-documented polymorphism at *Adh–2* produces both fast (toward the cathode) and slow allozymes of ADH–2 (Zouros, '73; Richardson et al., '77; Heed, '78; Batterham et al., '83b). The frequency of the fast allele ($Adh–2^{F}$) ranges from about 0.9–1.0 in Baja populations, whereas in mainland populations the slow allele ($Adh–2^{S}$) predominates, being present at frequencies ranging from about 0.7–1.0.

Drosophila ADH catalyzes the oxidation of primary and secondary alcohols to aldehydes and ketones and plays an important role both in detoxification and in providing nutrients for metabolism (Starmer et al., '77; Van Herrewege and David, '80; Geer et al., '88). *Drosophila* ADH, a member of the short-chain dehydrogenase/reductase (SDR) family of enzymes, differs from mammalian ADH in that it does not require zinc as a cofactor, does not breakdown methanol, and shows a preference for secondary alcohols, especially 2–propanol (Benach et al., 2001; Smilda et al., 2001). 2–propanol, however, has been termed a ''suicide'' substrate (e.g. Eisses, '97) because its oxidation produces acetone which inhibits the enzyme by forming a ternary complex with the coenzyme (NAD^{+}) and the enzyme (Schwartz et al., '79; Winberg and McKinley-McKee, '88; Benach et al., '99). The ternary complex is more electronegative than the native enzyme, resulting in the well-known phenomenon of ADH electrophoretic band interconversion produced after 2–propanol exposure or direct feeding of acetone to flies (Schwartz and Sofer, '76; Papel et al., '79; Anderson and McDonald, '81; also see Fig. 1).

The large differences in 2–propanol levels between host cacti utilized by subraces BI and BII of *D. mojavensis* (Starmer et al., '86; Fogleman and Heed, '89; Fogleman and Abril, '90), together with the known inhibitory effects of 2–propanol on ADH activity, has led to the view that 2–propanol may act as a potential selective force involved in maintaining the *Adh–2* allele frequency differences between subraces. According to Starmer et al. ('77), interactions among moderate air temperatures, low variation in pH and high 2–propanol levels in the pitaya agria host favor the F/F genotype in Baja; the higher air temperatures in Sonora, along with more variable pH and very low levels of 2–propanol in organpipe cactus rots favor the S/S genotype. Although the product of the $Adh–2^{F}$ allele is more temperature and pH sensitive than that of $Adh–2^{S}$ (Starmer et al., '77; Batterham et al., '83b), the physiological relevance of these differences, especially the different temperature sensitivities, is unclear (Batterham et al., '83b). Thus 2–propanol seems the most likely candidate as a selective factor. Consistent with this view is the finding that the $Adh–2^{F}$ allele in subrace BII of *D. mojavensis* is generally associated with a higher 2–propanol longevity response (Batterham et al., '82). Increased longevity in the presence of 2–propanol, however, was sometimes seen in subrace BI in which $Adh–2^{S}$ predominates (Batterham et al., '82). In addition, frequencies of the $Adh–2^{S}$ allele are relatively high in flies of subrace BI collected from the El Desemboque area in Sonora that utilize a small pocket of pitaya agria (Richardson et al., '77). It is unclear, however, whether the high frequency of the slow

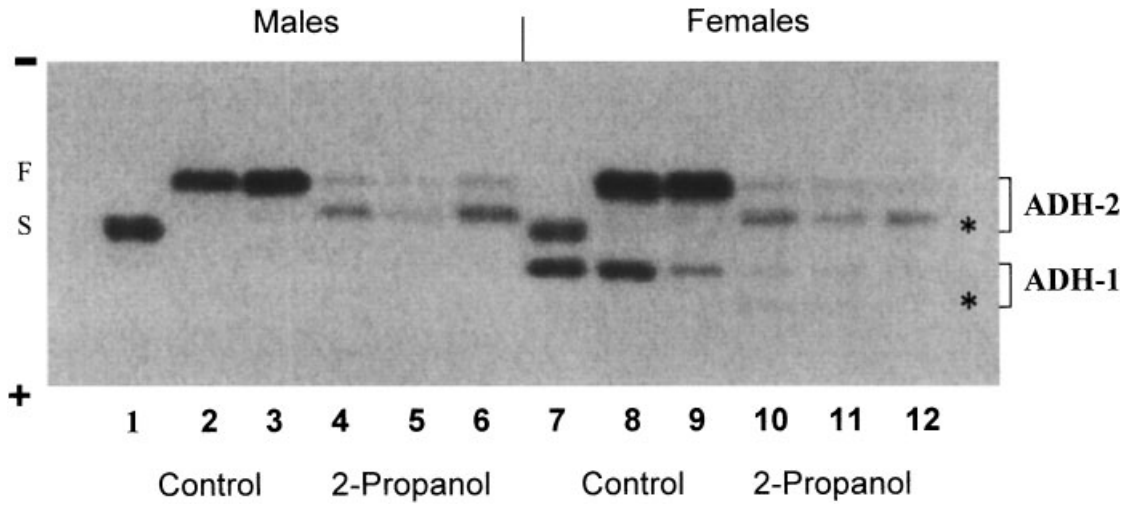

Fig. 1. Cellulose acetate electrophoresis of ADH isozymes of *Drosophila mojavensis* showing the effect of 24 h exposure to 2–propanol. Males, which express only ADH–2, are shown in lanes 1–6; females, which express both ADH–1 and ADH–2, are shown in lanes 7–12. Race BI flies from San Carlos, Sonora homozygous for $Adh\text{–}2^{S}$ are shown in lanes 1 and 7. All other lanes are race BII flies from Ensenada de los Muertos, Baja California Sur homozygous for $Adh\text{–}2^{F}$. Both isozymes show typical inhibition and band interconversion, labeled as * (barely visible for ADH–1), after 2–propanol exposure.

allele in this population is due to an absence of selection for $Adh\text{–}2^{F}$ by 2–propanol or is the result of immigration of flies using organpipe cactus rots in adjacent areas which experience little 2–propanol exposure.

The main focus of the present study was to test whether the F/F and S/S genotypes of ADH–2 show differences in inhibition after exposure to 2–propanol which might provide a basis for selection at the *Adh–2* locus. Mature male flies from seven different localities that were homozygous for either $Adh\text{–}2^{F}$ or $Adh\text{–}2^{S}$ were exposed to 2–propanol for 24 h; ADH–2 specific activity was then determined. Representatives of all described races of *D. mojavensis* were analyzed. To control for possible differences in genotype effects among localities and host plants, 2–propanol inhibition was studied in paired F/F and S/S genotypes in laboratory strains of *D. mojavensis* collected from the same locality [Grand Canyon, Arizona (race A) and Santa Catalina Island (race C)]. *Adh* expression differences between males and females were used to assess the effects on 2–propanol inhibition on ADH–1 activity in ovaries and total ADH activity (ADH–1 plus ADH–2) in whole female flies.

MATERIALS AND METHODS

Collection of flies

Both wild-caught and laboratory-reared flies were used. Adult *D. mojavensis* subrace BI from San Carlos, Sonora, Mexico were aspirated directly from natural organpipe cactus rot pockets, or from artificial baits prepared with organpipe tissue, into eight dram shell vials containing a culture medium of either cornmeal or banana/*Opuntia*. Isofemale strains, each derived from a single inseminated, wild-caught female, were established from collections made at the following areas: El Desemboque, Sonora (subrace BI that utilizes pitaya agria as a host), Ensenada de los Muertos, Baja California Sur (subrace BII), San Borja, Baja California [subrace BII from the Tucson *Drosophila* Species Stock Center, Center for Insect Science, University of Arizona (15081–1351.9)], Anza-Borrego Desert State Park, San Diego County, California (race A), Whitmore Canyon area near the Grand Canyon, Arizona (race A), and Santa Catalina Island, California (race C). Isofemale strains were then screened by electrophoresis (see below) to identify those strains that were homozygous for either $Adh\text{–}2^{F}$ or $Adh\text{–}2^{S}$ in order to utilize strains of the same host race, but of contrasting genotypes, for the inhibition experiments.

Electrophoresis

Cellulose acetate electrophoresis was used to screen strains of *D. mojavensis* for ADH–2 genotype, to confirm the presence of ADH–1 in larvae and ovaries, and to monitor ADH band interconversion after 2–propanol treatment.

Recently-eclosed flies were sorted by sex in the laboratory and held for at least seven days to assure that no ADH–1 activity remained in males. Individual flies were homogenized in 15 μl grinding buffer (10 mM Tris-HCl, 1.0 mM Na_2EDTA, 0.05 mM $NADP^+$; pH 7.5). Homogenates were centrifuged for 2 min at 10,000*g* and the supernatants were analyzed by electrophoresis on Titan III cellulose acetate plates (Helena Laboratories, Beaumont, TX) at room temperature for 15 min at 200 V using 0.025 M Tris, 0.192 M glycine buffer (pH 8.0); enzyme activity staining using 2–propanol as substrate followed the method of Hebert and Beaton ('89) with minor modifications.

2–propanol exposure

To expose flies from strains of known *Adh–2* genotype to 2–propanol vapor, a 7.5 × 3 cm piece of Kimwipe tissue was folded and placed into a vial containing banana/*Opuntia* culture medium. After pressing part of the tissue into the food, 100 μl of 2–propanol was applied to the tissue and then a thin (2 mm) disc sectioned from a compressed cotton plug was inserted into the vial to protect flies from direct contact with 2–propanol. Eight to twelve mature flies from each strain were placed into the vial, which was capped with a cotton plug. While little or no mortality was seen in any of the strains under these conditions, any flies that appeared damaged or unhealthy were discarded. After 24 h, flies were removed and prepared for assay of ADH activity.

Enzyme assays

For both control and 2–propanol-treated flies, 3–4 live individuals were aspirated into a 1.5 ml microcentrifuge tube and homogenized with a plastic pestle after adding 0.15 ml water. The homogenate was centrifuged for 2 min at 10,000*g*. ADH activity was determined immediately afterward on the supernatant fraction by following the increase in absorbance at 340 nm for 5 min at 28°C in an assay medium containing 0.052 M Tris-HCl (pH 8.0), 5.1 mM NAD^+, and 0.20 M 2–propanol in a final volume of 5.0 ml. A molar extinction coefficient for NADH of $5{,}200\,M^{-1}\,cm^{-1}$ was determined empirically for the assay conditions. Preliminary experiments and periodic monitoring confirmed that ADH reaction rates were linear over a 5 min period. Reactions were typically run in duplicate and started by addition of 0.05 ml of the supernatant fraction. For a few assays in which reaction rates were expected to be very low, single runs using 0.1 ml of the supernatant were conducted. All assays were usually repeated at least three times on separate groups of control and 2–propanol-treated males and females from each locality.

2–propanol inhibition was expressed as a simple percentage or proportion and calculated as the difference in ADH activity between control and 2–propanol treatment divided by the control. Mature males express only ADH–2, but enzyme activity in female flies includes both ADH–1 from the ovaries and ADH–2. To compare the effects of 2–propanol treatment on both isozymes separately, ovaries were dissected from females of subrace BI from San Carlos homozygous for the $Adh–2^S$ allele, and were assayed separately from the corresponding carcasses. Males from the same strain were also run for comparison of ADH–2 between sexes. Ovaries containing mature eggs were dissected from three groups of five females each of control and 2–propanol-treated flies and homogenized in 0.15 ml of water. The combined carcasses were also homogenized in 0.15 ml water. After centrifuging, ADH activity was determined on 0.1 ml of the supernatant as before. Protein was determined by the Hartree modification of the Lowry procedure (Hartree, '72) using bovine serum albumin as a standard.

Statistical analyses

Nested ANOVA was used to test for significant differences in ADH–2 activity by race, locality (nested within race), genotype (nested within locality) and 2–propanol exposure (nested within genotype). ADH–2 activity data were square root transformed before analysis. When testing for significant differences in inhibition of ADH by 2–propanol expressed as proportions, data were arcsine transformed before ANOVA was performed. Although a few data are presented on mixed genotypes, only data obtained from known F/F and S/S genotypes were used in the statistical analyses unless stated otherwise. A paired samples *t* test was used to determine whether 2–propanol exposure resulted in significant differences in activity in ADH–1 (ovaries) or ADH–2 (carcasses) of dissected females. Analyses were conducted in SYSTAT Version 9 and JMP IN version 4.0. Significance level was set at 0.05 unless stated otherwise.

RESULTS

Adh–2 in mature male flies

ADH–2 specific activities in control and 2–propanol-treated adult males of *D. mojavensis* from different geographic regions and different *Adh–2* genotypes are given in Table 2. ANOVA revealed a significant effect of race on control ADH–2 activity ($F_{3,\ 22}$=75.30; $P<0.0001$), but the nested effects of locality ($F_{3,\ 22}$=2.50; P=0.086) and genotype ($F_{2,\ 22}$=0.70; P=0.510) were not significant. Inspection of Table 2 suggested that the statistical significance of race resulted from low control ADH–2 activities in all genotypes of race A flies, a result also reported in an earlier study (Batterham et al., '83b). To confirm this, all race A data were removed and the ANOVA repeated. No significant differences were found in control activities among races ($F_{2,\ 15}$=2.27; P=0.140).

For all races and all *Adh–2* genotypes, 24 h exposure to 2–propanol resulted in substantial inhibition (46–92%) of ADH–2 activity (Table 2). The degree of inhibition was not related to the large differences in control ADH–2 activity described above. For example, 2–propanol inhibition in race C flies from Santa Catalina Island and race A flies from Anza-Borrego, both homozygous for the slow allele, was very similar (85 and 90%, respectively) although control activity was about four times higher in race C flies (Table 2). ANOVA indicated that there were significant differences in the effect of 2–propanol on ADH–2 activity among races ($F_{3,\ 44}$=94.08; $P<0.0001$), with less inhibition (46–60%) occurring in race BII flies from Baja that were homozygous for the fast allele of *Adh–2* than in races A, BI, and C (71–92% inhibition) comprising S/S, F/F, and mixed genotypes of *Adh–2*. The effect of 2–propanol on ADH–2 activity was significantly different for each race versus all the others, with the exception of race BI, in post hoc least squares means contrasts using an alpha value corrected to 0.0125 for multiple comparisons (race A vs. all: t_{60}=14.76, $P<0.0001$; race BI vs. all: t_{60}=0.89, P=0.380; race BII vs. all: t_{60}=9.93, $P<0.00001$; race C vs. all: t_{60}=2.95, P=0.005). No significant differences were found between S/S and mixed genotypes for control ADH–2 activity or degree of 2–propanol inhibition in race BI from San Carlos, or for 2–propanol inhibition in race A from Anza-Borrego, but control activity in mixed genotypes from Anza-Borrego was significantly lower than in the S/S homozygotes ($F_{1,\ 5}$=21.97; P=0.005).

Figure 1 shows that 2–propanol exposure resulted in typical ADH–2 electrophoretic band interconversion in both males and females of *D. mojavensis* subrace BII from Ensenada de los Muertos homozygous for *Adh–2*^{F}. 2–propanol-induced band interconversion was also seen in race BI flies from San Carlos homozygous for *Adh–2*^{S} (not shown). In addition, band interconversion occurred in ADH–1 in female flies of subrace BII (Fig. 1). The inhibitory effect of 2–propanol exposure on ADH activity is also evident from the relative decrease in activity staining in Figure 1.

Genotype (nested within locality and race) did not describe significant variance in either control

TABLE 2. Effect of 24 h 2-propanol exposure on ADH-2 activity in different geographic races and Adh-2 genotypes of adult male Drosophila mojavensis *determined at 28°C*

Race	Locality	Genotype	ADH-2 specific activity (±SE) (nmoles of NADH × mg protein^{-1} × min^{-1}		
			Control (*n*)	2-Propanol (*n*)	% Inhib.
A	Anza-Borrego, Calif.	S/S	34.2±1.9 (4)	3.3±1.9 (4)	90
A	Anza-Borrego, Calif.	Mixed[1]	14.6±4.2 (3)	1.2±0.2 (3)	92
A	Grand Canyon, Ariz.	S/S	26.4±6.7 (3)	7.6±1.7 (3)	71
A	Grand Canyon, Ariz.	F/F	34.5±1.1 (3)	9.1±1.5 (4)	74
BI	El Desemboque, Sonora	S/S	81.2±7.9 (3)	13.7±4.1 (3)	83
BI	San Carlos, Sonora	S/S	115.0±6.1 (2)[3]	27.7 (1)	76
BI	San Carlos, Sonora	Mixed[2]	117.5±20.5 (3)	21.7±2.3 (3)	82
BII	ENMU, Baja Calif. Sur	F/F	105.9±6.3 (4)	57.2±7.1 (4)	46
BII	San Borja, Baja Calif.	F/F	124.9±13.3 (5)	49.7±10.8 (5)	60
C	Santa Catalina Is., Calif.	F/F	121.0±10.6 (4)	15.8±4.3 (4)	87
C	Santa Catalina Is., Calif.	S/S	123.4±8.5 (3)	18.8±1.3 (3)	85

[1]Frequency of *Adh-2*^{F}=0.56 (*n*=16).
[2]Frequency of *Adh-2*^{S}=0.76 (*n*=67).
[3]Mean and range of two determinations.

($F_{2,\ 22}$=0.70; P=0.510) or 2–propanol effect on ADH–2 activity ($F_{2,\ 44}$=0.70; P=0.500). In paired comparisons of F/F and S/S *Adh–2* genotypes in male flies from the Grand Canyon (race A) and Santa Catalina Island (race C) the F/F genotype showed high 2–propanol inhibition [87% (race A) and 74% (race C)] that was not significantly different from that seen with the S/S genotype (race A: t_{11}=1.02, P=0.315; race C: t_{12}=0.61, P=0.545; Table 2). No significant difference in mean control ADH–2 activity was seen between F/F and S/S genotypes in either race A (t_4=1.17; P=0.267) or race C (t_5=0.20; P=0.846) flies.

Adh–1 and Adh–2 in mature female flies

The expression of both *Adh–1* (ovaries) and *Adh–2* (carcasses) in females of *D. mojavensis* allowed also for testing of the effect of 2–propanol exposure on the products of two separate loci simultaneously on the same individuals. ADH–2 specific activity in carcasses of dissected S/S females of subrace BI from San Carlos (104.3 ± 6.5 nmoles NADH x mg protein^{-1} × min^{-1}; n=3; Fig. 2) was not significantly different from control values obtained from S/S and mixed genotype males from the same locality ($F_{1,\ 6}$=0.59; P=0.470; Table 2). Also, 2–propanol exposure inhibited ADH–2 in female carcasses by 75% (Fig. 2) which was statistically significant (t_2=16.68; P=0.004) and not significantly different from the ADH–2 inhibition seen in males from San Carlos ($F_{1,\ 5}$=4.05; P=0.100; Table 2). ADH–1 specific activity in female ovaries, however, was only inhibited 39% by 2–propanol (Fig. 2), about half the inhibition seen with ADH–2, but the difference in activities between control and 2–propanol treatment was not significant (t_2=3.21; P=0.085). The difference in inhibitory effect of 2–propanol on ADH–1 and ADH–2 in females was significant ($F_{1,\ 4}$=17.79; P=0.014). Before being standardized to protein, ADH activities in carcasses and their respective ovaries were summed and used to calculate that about 70–75% of the total ADH activity in control female flies was contributed by ADH–2.

Table 3 shows the effect of 2–propanol exposure on total ADH (ADH–1 and ADH–2 combined) specific activities in whole body adult females of *D. mojavensis* with different *Adh–2* genotypes and from different geographic regions. While the data in Tables 2 and 3 are not directly comparable because they are confounded by the presence of ADH–1 in female ovaries, as well as by the

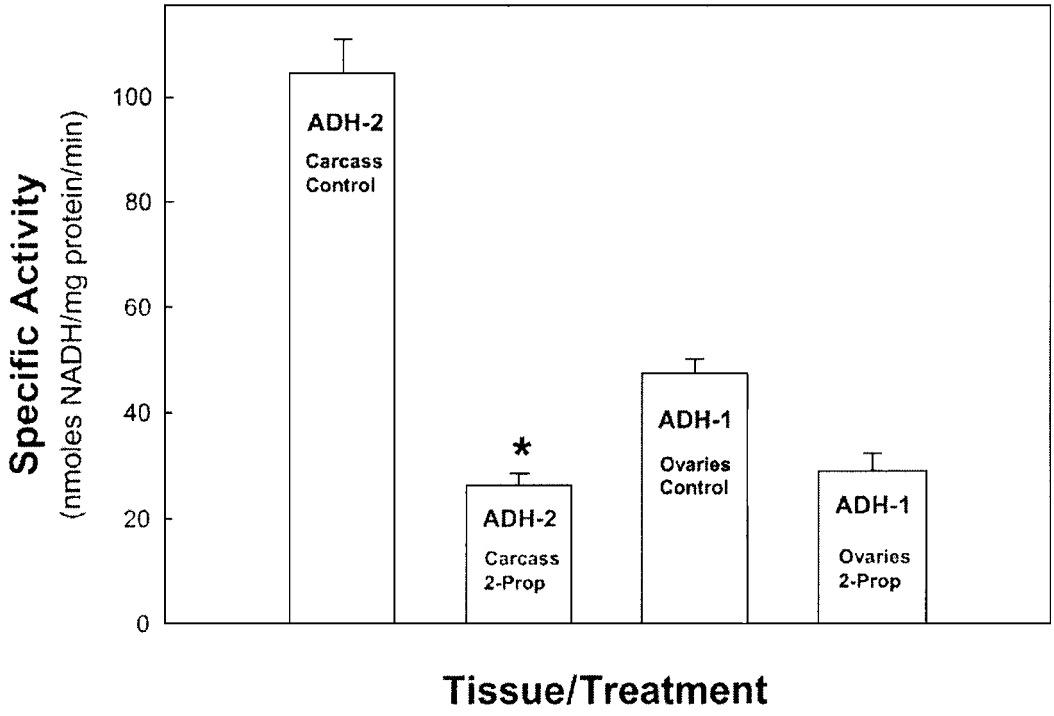

Fig. 2. Effect of 24 h 2–propanol exposure on ADH–2 activity in carcasses and ADH–1 activity in ovaries of dissected females of *Drosophila mojavensis* race BI from San Carlos, Sonora homozygous for *Adh–2*^{S}. Values are means (n=3) with standard errors shown as vertical bars. *, significantly different from control.

TABLE 3. Effect of 24 h 2-propanol exposure on total ADH activity (ADH-1 and -2) in different geographic races and Adh-2 genotypes of female Drosophila mojavensis *determined at 28°C*

Race	Locality	Genotype	ADH specific activity (±SE) (nmoles of NADH × mg protein^{-1} × min^{-1})		
			Control (*n*)	2-Propanol (*n*)	% Inhib.
A	Anza-Borrego, Calif.	S/S	23.0±4.7 (4)	0.0±0.0 (4)	100
A	Anza-Borrego, Calif.	Mixed[1]	9.9±1.7 (3)	2.3±0.2 (3)	77
A	Grand Canyon, Ariz.	S/S	17.8±3.0 (3)	2.9±1.1 (3)	84
A	Grand Canyon, Ariz.	F/F	16.4±1.8 (3)	1.6±0.5 (3)	90
BI	El Desemboque, Sonora	S/S	51.7±3.8 (3)	20.6±0.1 (3)	60
BI	San Carlos, Sonora	S/S	73.2±15.1 (2)[3]		
BI	San Carlos, Sonora	Mixed[2]	66.3±4.7 (3)	16.2±2.5 (3)	76
BII	ENMU, Baja Calif. Sur	F/F	70.9±8.8 (4)	43.7±5.3 (3)	38
BII	San Borja, Baja Calif.	F/F	104.4±21.9 (4)	60.1±13.3 (4)	42
C	Santa Catalina Is., Calif	F/F	121.9±10.3 (4)	22.7±4.3 (4)	81
C	Santa Catalina Is., Calif	S/S	98.5±4.4 (3)	21.5±4.2 (3)	78

[1]Frequency of $Adh\text{-}2^{F}$=0.56 (n=16).
[2]Frequency of $Adh\text{-}2^{S}$=0.76 (n=67).
[3]Mean and range of two determinations.

differential inhibitory effect of 2–propanol on the two isozymes, they are presented to show that the same trends are seen in both males (ADH–2 only) and females. Total ADH specific activities were lower in females of race A than in the other races, and subrace BII females homozygous for $Adh\text{–}2^{F}$ were less susceptible to 2–propanol inhibition (38–42%) than flies from the other races (60–100%). Again, no statistically significant genotype differences were seen in the high degree of 2–propanol inhibition (78–90%) between F/F and S/S genotypes of ADH–2 in race A from the Grand Canyon (t_{10}=0.46; P=0.650) and race C from Santa Catalina Island (t_{12}=1.10; P=0.277). Assuming that ADH–1 inhibition is the same in both genotypes, these results provide further support for the view that 2–propanol is not directly selecting for the $Adh\text{–}2^{F}$ allele.

During the genotyping of flies for ADH–2 it was found that the typical ADH–1 electrophoretic band observed in females of *D. mojavensis*, including those of race A from Anza-Borrego, was missing in one line of F/F race A females (n=15) and third instar larvae (n=5), and in one line of S/S race A females (n=2), from the Grand Canyon. Additional experiments revealed that the ovaries of three females from the F/F line expressed ADH–1, but that it was more electropositive than typical ADH–1, migrating to a position similar to that of both $ADH\text{–}2^{S}$ and the satellite band of $ADH\text{–}2^{F}$ (not shown; see Fig. 1 for reference). These observations suggest that a putative fast allele of *Adh–1* is present in this population, although previous studies on *D. mojavensis*, none of which included flies from the Grand Canyon, have suggested that the *Adh–1* locus is monomorphic (Heed, '78; Matzkin and Eanes, 2003). Sequencing studies on these two Grand Canyon lines of *D. mojavensis* are planned in order to determine the molecular basis for the difference in electrophoretic behavior.

DISCUSSION

Although Baja populations of *D. mojavensis* (subrace BII) homozygous for the fast allele of *Adh–2* showed less 2–propanol inhibition than the other geographic races, our results do not support the hypothesis that high 2–propanol level alone in the pitaya agria host cactus is a selective force favoring the $Adh\text{–}2^{F}$ allele. High 2–propanol inhibition was seen in both F/F and S/S genotypes of *Adh–2* in paired comparisons of flies from both the Grand Canyon (race A) and Santa Catalina Island (race C) and, most important, the degree of inhibition was not statistically different between genotypes from each locality. In addition, the F/F genotype from all regions showed the typical electrophoretic band interconversion associated with the formation of an inhibitory ternary complex between enzyme, acetone, and NAD^{+}. Thus, there is no evidence that the gene product of $Adh\text{–}2^{F}$ responds differently to 2–propanol exposure than the product of $Adh\text{–}2^{S}$. Owing to the low frequency of $Adh\text{–}2^{S}$ in Baja, none of the laboratory lines of subrace BII were homozygous for the slow allele. If the F/F genotype alone was responsible for reduced 2–propanol inhibition, however, the effect should not be limited to F/F

flies from a single geographic area, unless some other factor, such as an amino acid substitution(s) that has no effect on net charge of the protein, is responsible. We suggest that the reduced inhibitory effect of 2–propanol on ADH–2 in subrace BII from Baja, and the increased longevity of BII flies when exposed to 2–propanol (Batterham et al., '82), is not directly related to the F/F genotype and its characteristic amino acid substitutions (Matzkin and Eanes, 2003), but rather is due to some other factor acting alone or together with *Adh–2*^{F}. Consistent with this notion is the observation that high frequencies of *Adh–2*^{F} are not specifically associated with utilization of pitaya agria. The frequency of *Adh–2*^{F} is 0.70 (n=60) in race C flies from Santa Catalina Island (Hocutt, 2000) and 0.55 (n=124) in race A flies from the Anza-Borrego area (Cleland et al., '96). Both of these races utilize alternative host plants, *Opuntia littoralis* and *Ferocactus cylindraceus*, respectively (Table 1). Levels of 2–propanol are much lower in *O. stricta* than in pitaya agria (Starmer et al., '86); there is no information currently available on volatile concentrations of rots in these other hosts. In race A flies from the Grand Canyon the frequency of *Adh–2*^{F} is lower than in race A flies from the southern California desert (f=0.34; n=41) (Cleland et al., '96) but higher than typically seen in BI flies.

There are also other considerations that argue against the *Adh–2* locus being the focus of selection by 2–propanol. Due to their restriction to the pitaya agria rot pocket, it is likely that subrace BII larval stages, which express predominately *Adh–1* during the first two instars, are exposed to 2–propanol vapors to a greater extent than the vagile adult stage in which mainly *Adh–2* is expressed. Batterham et al. ('82) have shown that total ADH specific activity increases markedly during the larval stages, but then shows a dramatic decrease after eclosion. ADH–1 from ovaries of subrace BI flies shows significantly less 2–propanol inhibition than ADH–2 (Fig. 2), but no data are available on 2–propanol inhibition of ADH–1 in pure lines of the other races. However, preliminary results of crosses of BI flies from El Desemboque homozygous for *Adh–2*^{S} and BII flies from Ensenada de los Muertos homozygous for *Adh–2*^{F} also showed a reduced inhibitory effect of 2–propanol exposure on ADH–1 in ovaries of F_1 females. No 2–propanol inhibition was seen in F_1 females obtained from the cross of BI females with BII males, and only 30% inhibition was seen in the cross of BI males with BII females (n=2 for each cross). ADH–2 in carcasses of the F/S heterozygotes from the two crosses was inhibited by 55 and 60%, respectively. Matzkin and Eanes (2003) provide evidence for adaptive protein evolution at the *Adh–1* locus associated with the host shift that occurred during the divergence of *D. mojavensis* from *D. arizonae* approximately 2.4 million years ago, again suggesting that changes in the chemical composition of the host environment may exert a greater selective effect on ADH–1 in larvae than on ADH–2 in adults.

The fact that *Drosophila* ADH shows a substrate preference for secondary alcohols such as 2–propanol over ethanol has always seemed something of an oddity, especially for those species such as *D. melanogaster* that are routinely subjected to high ethanol levels in their environment and require ADH to breakdown ethanol both for detoxification and as an energy source (Van Herrewege and David, '80; Geer et al., '88). Kinetic and structural studies on ADH in *D. lebanonensis* and *D. simulans* have provided an explanation for this unique characteristic by showing that the active site is bifurcated and ideally suited for secondary alcohols (Benach et al., 2001; Smilda et al., 2001). However, species such as *D. mojavensis* from Baja that are subjected to elevated concentrations of 2–propanol in their host cactus seem particularly susceptible to the inhibitory effects of acetone produced by oxidation of 2–propanol by ADH. One line of reasoning is that high ADH inhibition in a 2–propanol environment would actually be beneficial because it would reduce the production of highly toxic acetone and favor survival (Anderson and McDonald, '81). Guillén et al. ('87), however, found that mortality was less after 2–propanol exposure in *D. melanogaster* when ADH activity was the highest. They conclude that if there is some type of adaptive benefit of acetone production, other enzymes must be involved. In any event, there is no known physiological benefit of oxidation of 2–propanol to acetone.

The molecular basis and physiological consequences of the dramatically reduced ADH–2 specific activity in control race A male flies of both F/F and S/S genotypes compared to the other races (Table 2) remain to be determined. Total ADH activity (ADH–1 plus ADH–2) also was very low in whole-body homogenates of race A females (Table 3). The 3– to 4–fold lower ADH–2 activity in race A males agrees with the 3.6–fold lower ADH–2 activities seen by Batterham et al. ('83b) for both

genotypes in mature males of *D. mojavensis* race A from the Anza-Borrego area (Vallecito, Calif.) compared with race BII flies from Isla San Esteban in the Gulf of California. Although 2–propanol was used as substrate in both studies, Batterham et al. ('83b) used a partially purified enzyme preparation which indicates that the low activity in race A reported in the present study was not the result of an inhibitory substance in the crude homogenates but was rather an inherent property of the ADH–2 molecule. In males of *D. pachea*, another cactophilic drosophilid from the Sonoran Desert, ADH activity is present in larvae and pupae, but it is lost several days after eclosion, although activity can be induced in the laboratory by exposing flies to a variety of alcohols (Pfeiler and Markow, 2001, 2003). Wild-caught adult males of *D. pachea* usually show no ADH activity staining. These results again point to the importance of ADH during the larval stages and suggest that expression of the enzyme in adults can be plastic without producing any major detrimental effects on fitness.

The molecular basis for the charge difference characterizing the fast/slow polymorphism at *Adh–2* in *D. mojavensis* has been shown to result from a single amino acid substitution (arginine to serine in $Adh\text{–}2^{S}$), with a total of five amino acid replacements separating $Adh\text{–}2^{F}$ and $Adh\text{–}2^{S}$ (Matzkin and Eanes, 2003). The well-documented fast/slow *Adh* polymorphism in *D. melanogaster* (Kreitman, '83) and in the olive fruit fly *Bactrocera oleae* (Goulielmos et al., 2003) are also known to result from a single amino acid substitution that produces a net charge difference. Polymorphism in *D. melanogaster* has been studied extensively, so comparisons with *D. mojavensis* are often made (e.g., Starmer et al., '77). It is important to point out, however, that the terms ''fast'' and ''slow'' are not equivalent with respect to charge characteristics in interspecific comparisons. In *D. melanogaster*, the reference electrode has historically been taken as the anode and a threonine to lysine substitution produces a more electropositive allozyme that is denoted ''slow.'' In *D. mojavensis* and *B. oleae* the cathode has been assigned as the reference electrode (Starmer et al., '77; Goulielmos et al., 2003, present study) and the ''slow'' allele is more electronegative than the ''fast'' allele.

In conclusion, the lack of significant differences in 2–propanol-induced ADH–2 inhibition between $Adh\text{–}2^{S}$ and $Adh\text{–}2^{F}$ genotypes of *D. mojavensis* from both Santa Catalina Island and the Grand Canyon does not support the hypothesis that 2–propanol is a selective force acting directly on, and favoring, the fast allele. Other explanations for the temporal stability of high frequencies $Adh\text{–}2^{F}$ in Baja populations of *D. mojavensis*, the lower 2–propanol sensitivity of ADH–2 in Baja flies homozygous for $Adh\text{–}2^{F}$, and the increased longevity reported for Baja flies homozygous for $Adh\text{–}2^{F}$ (Batterham et al., '82) must be sought.

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LITERATURE CITED

Anderson SM, McDonald JF. 1981. Effect of environmental alcohol on in vivo properties of *Drosophila* alcohol dehydrogenase. Biochem Genet 19:421–430.

Atkinson PW, Mills LE, Starmer WT, Sullivan DT. 1988. Structure and evolution of the *Adh* genes of *Drosophila mojavensis*. Genetics 120:713–723.

Batterham P, Starmer WT, Sullivan DT. 1982. Biochemical genetics of the alcohol longevity response of *Drosophila mojavensis*. In: Barker JSF, Starmer WT, editors. Ecological Genetics and Evolution: The Cactus-Yeast-*Drosophila* Model System. Sydney: Academic Press. p 307–321.

Batterham P, Lovett JA, Starmer WT, Sullivan DT. 1983a. Differential regulation of duplicate alcohol dehydrogenase genes in *Drosophila mojavensis*. Dev Biol 96:346–354.

Batterham P, Gritz E, Starmer WT, Sullivan DT. 1983b. Biochemical characterization of the products of the *Adh* loci of *Drosophila mojavensis*. Biochem Genet 21:871–883.

Benach J, Atrian S, Gonzàlez-Duarte R, Ladenstein R. 1999. The catalytic reaction and inhibition mechanism of *Drosophila* alcohol dehydrogenase: observation of an enzyme-bound NAD-ketone adduct at 1.4 Å resolution by X-ray crystallography. J Mol Biol 289:335–355.

Benach J, Atrian S, Ladenstein R, Gonzàlez-Duarte R. 2001. Genesis of *Drosophila* ADH: the shaping of the enzymatic activity from a SDR ancestor. Chemico-Biological Interactions 130–132:405–415.

Cleland S, Hocutt GD, Breitmeyer CM, Markow TA, Pfeiler E. 1996. Alcohol dehydrogenase polymorphism in barrel cactus populations of *Drosophila mojavensis*. Genetica 98:115–117.

Eisses KTh. 1997. The influence of 2–propanol and acetone on oviposition rate and oviposition site preference for acetic acid and ethanol of *Drosophila melanogaster*. Behav Genet 27:171–180.

Fogleman JC, Abril JR. 1990. Ecological and evolutionary importance of host plant chemistry. In: Barker JSF, Starmer WT, MacIntyre RJ, editors. Ecological and Evolutionary Genetics of *Drosophila*. New York: Plenum Press. p 121–143.

Fogleman JC, Heed WB. 1989. Columnar cacti and desert *Drosophila*: the chemistry of host plant specificity. In:

Schmidt J, editor. Special biotic relationships in the arid southwest. Albuquerque: University of New Mexico Press. p 1–24.

Geer BW, McKechnie SW, Bentley MM, Oakeshott JG, Quinn EM, Langevin ML. 1988. Induction of alcohol dehydrogenase by ethanol in *Drosophila melanogaster*. J Nutr 118: 398–407.

Goulielmos GN, Cosmidis N, Theodorakopoulou ME, Loukas M, Zouros E. 2003. Tracing the history of an enzyme polymorphism: the case of alcohol dehydrogenase–2 (*Adh–2*) of the olive fruit fly *Bactrocera oleae*. Mol Biol Evol 20:293–306.

Guillén E, Sánchez-Cañete FJS, Garrido JJ, Dorado G, Barbancho M. 1987. Intergenotypic effect of isopropanol ingestion in the further detoxification of ethanol and isopropanol in *Drosophila melanogaster*. Heredity 59: 405–411.

Hartree EF. 1972. Determination of protein: a modification of the Lowry method that gives a linear response. Analyt Biochem 48:422–427.

Hebert PDN, Beaton MJ. 1989. Methodologies for allozyme analysis using cellulose acetate electrophoresis. Beaumont, Texas: Helena Laboratories.

Heed WB. 1978. Ecology and genetics of Sonoran Desert *Drosophila*. In: Brussard PF, editor. Ecological Genetics: The Interface. New York: Springer-Verlag. p 109–126.

Heed WB, Mangan RL. 1986. Community ecology of the Sonoran Desert *Drosophila*. In: Ashburner M, Carson HL, Thompson, Jr. JN, editors. The genetics and biology of *Drosophila,*Vol. 3e. London: Academic Press. p 311–345.

Hocutt GD. 2000. Reinforcement of premating barriers to reproduction between *Drosophila arizonae* and *Drosophila mojavensis*. Ph.D. Thesis: Arizona State University, Tempe.

Kreitman M. 1983. Nucleotide polymorphisms at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature 304:412–416.

Markow TA, Hocutt GD. 1998. Reproductive isolation in Sonoran desert *Drosophila*: testing the limits of the rules. In: Howard DJ, Berlocher SH, editors. Endless Forms: Species and Speciation. New York: Oxford University Press. p 234–244.

Matzkin LM, Eanes WF. 2003. Sequence variation of alcohol dehydrogenase (*Adh*) paralogs in cactophilic Drosophila. Genetics 163:181–194.

Mettler LE. 1963. *D. mojavensis baja*, a new form in the mulleri complex. Drosophila Information Service 38:57–58.

Papel I, Henderson M, Van Herrewege J, David J, Sofer W. 1979. *Drosophila* alcohol dehydrogenase activity in vitro and in vivo: effects of acetone feeding. Biochem Genet 17:553–563.

Pfeiler E, Markow TA. 2001. Loss of expression of alcohol dehydrogenase in adult males of *Drosophila pachea*. Biochem Genet 39:139–144.

Pfeiler E, Markow TA. 2003. Induction of suppressed ADH activity in *Drosophila pachea* exposed to different alcohols. Biochem Genet 41:413–426.

Richardson RH, Smouse PE, Richardson ME. 1977. Patterns of molecular variation. II. Associations of electrophoretic mobility and larval substrate within species of the *Drosophila mulleri* complex. Genetics 85:141–154.

Ruiz A, Heed WB, Wasserman M. 1990. Evolution of the *mojavensis* cluster of cactophilic *Drosophila*, with descriptions of two new species. J Hered 81:30–42.

Schwartz M, Sofer W. 1976. Diet-induced alterations in distribution of multiple forms of alcohol dehydrogenase in *Drosophila*. Nature 263:129–131.

Schwartz M, O'Donnell J, Sofer W. 1979. Origin of the multiple forms of alcohol dehydrogenase from *Drosophila melanogaster*. Arch Biochem Biophys 194:365–378.

Smilda T, Kamminga AH, Reinders P, Baron W, Van Hylckama Vlieg JET, Beintema JJ. 2001. Enzymic and structural studies on *Drosophila* alcohol dehydrogenase and other short-chain dehydrogenases/reductases. J Mol Evol 52:457–466.

Starmer WT, Heed WB, Rockwood-Sluss ES. 1977. Extension of longevity in *Drosophila mojavensis* by environmental ethanol: Differences between subraces. Proc Natl Acad Sci USA 74:387–391.

Starmer WT, Barker JSF, Phaff HJ, Fogleman JC. 1986. Adaptations of *Drosophila* and yeasts: their interactions with the volatile 2–propanol in the cactus-microorganism-*Drosophila* model system. Aust J Biol Sci 39:69–77.

Van Herrewege J, David JR. 1980. Alcohol tolerance and alcohol utilization in *Drosophila*: partial independence of two adaptive traits. Heredity 44:229–235.

Winberg J-O, McKinley-McKee JS. 1988. *Drosophila melanogaster* alcohol dehydrogenase. Biochemical properties of the NAD^+-plus-acetone-induced isoenzyme conversion. Biochem J 251:223–227.

Zouros E. 1973. Genic differentiation associated with the early stages of speciation in the *mulleri* subgroup of *Drosophila*. Evolution 27:601–621.