Extension of longevity in *Drosophila mojavensis* by environmental ethanol: Differences between subraces

(alcohol dehydrogenase/isoenzyme thermostability/environmental heterogeneity)

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**ABSTRACT** *Drosophila mojavensis* adults, which breed and feed on necrotic cacti, show an increase in longevity when exposed to atmospheric ethanol. The increase in longevity is accompanied by retention of mature ovarioles and is independent of diet. Differences in longevity among strains from different localities were detected for females. Strains from Arizona and Sonora, Mexico, showed the greatest increase in longevity, while strains from Baja California, Mexico, showed the least increase. These differences may be controlled by the alcohol dehydrogenase locus, the octanol dehydrogenase locus, and modifier genes, because the adult response is correlated with the frequency of alcohol dehydrogenase alleles, as well as with second chromosomal inversions containing the octanol dehydrogenase locus. The longevity response is also consistent with the more uneven distribution and availability of the host plant in Arizona and Sonora, Mexico.

Strains from Arizona and Sonora, Mexico, have a high frequency of *Adh-S*, the allele whose product is heat and pH tolerant. The host plant, organpipe cactus, exhibits extremes in temperature and pH in the same geographic region. Strains from Baja California, Mexico, possess a high frequency of *Adh-F*, whose product is heat and pH sensitive. The substrate in this region, agria cactus, has moderate temperature and pH extremes and contains relatively high concentrations of isopropanol. Isopropanol is presumably a selective agent favorable to *Adh-F*. The environmental heterogeneity that is proposed for maintaining the alleles at the alcohol dehydrogenase locus is the interaction of substrate alcohol content with temperature and pH. Substrates that do not contain appreciable amounts of isopropanol and are exposed to high temperatures and exhibit variable pH favor *Adh-S*, while substrates containing isopropanol and having moderate temperatures and pH favor *Adh-F*.

The tolerance of *Drosophila melanogaster* to environmental alcohol and the maintenance of genetic polymorphism at the alcohol dehydrogenase (ADH) locus are presently under intense investigation. Studies have focused on the relationship of the ADH gene and genotypes to ethanol tolerance of *D. melanogaster* populations found in various parts of the world (1-7). Furthermore, the gene product of the ADH locus has been studied (3, 8-10) and characterized with respect to specific activity, substrate specificity, heat stability, and pH optima and sensitivity.

Genetic modification of ADH activity has been suggested (2, 7, 9, 11, 12). The general picture that emerges is that the alleles at the ADH locus are under the influence of natural selection, yet subject to background modification when environmental stress is great.

The present paper demonstrates a diet-independent increase in longevity in *Drosophila mojavensis* Patterson and Crow on exposure to alcohol vapors. The longevity response is correlated with allele frequencies at the ADH locus. Environmental variables show correlations with the physico-chemical properties of the ADH alleles.

*Drosophila mojavensis* is known to breed and feed primarily on the fermenting stems of the giant cacti *Machaerocereus gummosus* (Engelm.) Brittt. and Rose (agria) and *Lemaireocereus thurberi* (Engelm.) Brittt. and Rose (organpipe) (13). *Drosophila mojavensis* prefers the former host in Baja California, Mexico, and the latter cactus in Arizona and Sonora, Mexico. Mettler (14) subdivided *D. mojavensis* into two races; *D. mojavensis* race A, confined to desert areas of southern California, and *D. mojavensis* race B, found in southern Arizona and Sonora and Baja California, Mexico. Zouros (15) established two subraces of *D. mojavensis* race B based on electrophoretic allelic frequency differences at three of five polymorphic loci. The differences were most pronounced for the ADH locus. The allele *Adh-S* was found with frequency of 0.84 or higher in *D. mojavensis* subrace B1 (Arizona and Sonora), while this allele had frequency of 0.09 or lower for *D. mojavensis* subrace BII (Baja California and the islands of the Gulf of California). Independent observations (16) showed similar differences. The subdivision of *D. mojavensis* race B into subraces is supported by Johnson (17). Johnson found populations of subrace I to be predominantly monomorphic, while subrace II in central Baja California exhibited a high degree of polymorphism in inversions of the second and third chromosomes.

A recent survey (18) of the yeasts associated with desert *Drosophila* and their host plants indicates adult *D. mojavensis* utilize *Pichia membranaefaciens* Hansen and *Tolypopsis sonorensis* Miller et al. *Tolypopsis sonorensis* is specific for rotting cacti (19); of those cacti studied, it is in the host plants of *D. mojavensis* that this yeast is most frequently found. Furthermore, it is the only yeast recovered from cactus substrates that ferments D-glucose. This implies the substrates of *D. mojavensis* could contain alcohol as a fermentation product. In addition other organisms, such as bacteria, may contribute to the alcohol content of the substrate.

**METHOD**

Approximately 1 g of bleached cotton (Padco, nonsurgical) is placed in an 8 dram (30 ml) shell vial to which 10 ml of a test solution is added (e.g., H₂O, 1.0% ethanol, etc.). In a second 8 dram shell vial a given number of adults (usually ten) are placed and the top is covered with one layer of cheesecloth. This vial is inverted and sealed to the first vial with a strip of Parafilm and Scotch brand tape. The seal is made as air-tight as possible and the system is essentially closed, with adults exposed to a constant amount of atmospheric ethanol (or water vapor in controls) in equilibrium with the liquid phase found in the lower
cotton-containing vial. The tests were conducted at 25°C. The adults are scored morning, midday, and evening for the number living and dead. The resulting observation is the longevity of individual adults.

**EXPERIMENTS AND RESULTS**

**Experiment 1.** The first experiment was carried out with 20 (10 of each sex) adult *D. mojavensis* (A570, El Barril, Baja California, Mexico) in treatment vials containing 0.0, 0.5, 1.0, 2.0, 4.0, and 8.0% (vol/vol) ethanol. The observation (hours of life) was transformed to ln (hr) to ensure homogeneity of variances for an analysis of variance. The analysis indicated a significant ($\alpha = 0.001$) effect of ethanol on the longevity of the adults. Flies in treatment vials of 1.0, 2.0, and 4.0% ethanol showed a marked increase in longevity when compared to those in the control vial (see Fig. 1). It was found that the longest-lived females (approximately 6 days) in the water controls had resorbed their eggs, whereas the longest-lived females (approximately 14 days) in the 4.0% ethanol vials had intact ovarioles and eggs. Subsequent replica experiments utilizing different strains of *D. mojavensis* confirmed these results. A single experiment with *D. melanogaster* (Oregon-R) revealed a response to ethanol fumes similar to that of *D. mojavensis*. *D. melanogaster* adults live longer in ethanol treatment vials of 2.0 and 4.0% than in the 0.0, 0.5, 1.0, and 8.0% vials.

**Experiment 2.** In order to test the hypothesis that the increased longevity is dependent upon the yeasts present in the gut of the adult at the time of isolation, two axenic strains of *D. mojavensis* were established (A300 Navojoa and A240 Hermosillo, both localities in Sonora, Mexico). Flies of both strains were divided into three groups, each subjected to 1 day of feeding on: (i) original dead yeast used in axenic culture, (ii) live *Candida krusei* (Cast.) Berkhourt (75-220.1) growing on YMA (yeast extract, malt extract, agar—Difco), and (iii) live *Kloeckera apiculata* (Rees emend. Klocker) Janke (75-90.1) growing on YMA. *Candida krusei* is frequently found associated with drosophilas (20). It is capable of growth on vitamin-free medium and has rapid growth when ethanol is the sole carbon source. *Kloeckera apiculata* is frequently found associated with drosophilas but cannot live on vitamin-free medium and does not utilize ethanol when it is supplied as the sole carbon source (21). Therefore, the feeding of *K. apiculata* affords a second control for assessing the ability of *C. krusei* in extending the longevity of the adults in an ethanol atmosphere. Ten adults (equal numbers of each sex) from each of the three groups of fed flies were placed in treatment vials of 0.0, 2.0, and 4.0% ethanol. All vials, cotton, cheesecloth, and treatment solutions were sterile at the beginning of the experiment. The experiment was conducted with both strain A300 and strain A240.

The remaining adults in the 4.0% ethanol experiment with strain A240 were sacrificed after 6 days and assayed for external and internal yeast flora. Adults from the original fed treatment group (i) had no associated yeasts, whereas adults from *C. krusei* (iii) and *K. apiculata* (iii) fed groups yielded colonies of the yeast they were given initially. The replicated experiment using strain A300 was carried out to completion. The results indicated no differences between vials within treatment groups; therefore replicates were pooled. An analysis of the pooled data is given in Table 1 and depicted in Fig. 2. The interaction between ethanol treatment and feeding treatment is significant ($\alpha = 0.05$) and obviously due to the response of the *C. krusei*-fed flies as compared to the other two (see Fig. 2). In any event, the differences are not striking and the significant positive response of the flies fed dead yeast negates the hypothesis that live yeasts are responsible for the observed significant increase in longevity for flies exposed to ethanol fumes.

**Experiment 3.** Seven laboratory strains of *D. mojavensis* from Baja California, Mexico, and six strains from Sonora, Mexico, and southern Arizona were utilized in an experiment to evaluate sex and subrace differences. Collection sites are shown in Fig. 3. Starch gel electrophoresis was used to measure the frequency of Adh-F for each strain. Ten individuals of the same age (4 days) were placed in treatment vials of 0.0, 2.0, and 4.0% ethanol, each strain and sex separately. The collection localities, frequency of inversions, frequency of Adh-F, and mean longevity for the sexes of each strain are given in Table 2.

In order to correct for inherent differential longevity among strains in control vials, the mean longevities of the 2.0 and 4.0% treatments were divided by the mean longevity of the corresponding control (0.0% treatment) vial. The average longevity for all controls was 190.32 hr (7.93 days). The relative increase in longevity for each strain was used in a 3-way analysis of variance (Table 3). The significant subrace by sex interaction is a result of the males of the Baja subrace having a larger relative longevity than the females (1.59 and 1.30, respectively), while the males of the Arizona–Sonora subrace have a smaller

![Figure 1](image-url)  
**Fig. 1.** Mean longevities given in ln (hr) for *D. mojavensis* exposed to increasing concentrations of atmospheric ethanol.
Table 2. The collection localities, mean longevity of each treatment group, frequency of inversions on the second and third chromosomes, and frequency of Adh-F for D. mojavensis strains used in Experiment 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Locality</th>
<th>Mean longevity in days for ethanol treatments</th>
<th>Frequency of inversions on second chromosome</th>
<th>Frequency of ST arrangement on third chromosome</th>
<th>Frequency of Adh-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>A350</td>
<td>San Borja, Baja</td>
<td>0.0% 8.69 7.17 13.70 11.44 11.16 8.73</td>
<td>0.53 0.40 0.06 0.0 0.29 1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A420</td>
<td>Punta Prieta, Baja</td>
<td>2.0% 9.26 6.98 17.51 11.81 17.46 13.66</td>
<td>0.44 0.18 0.36 0.0 0.04 1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A428</td>
<td>Punta Escandido, Baja</td>
<td>4.0% 12.06 8.58 14.68 14.30 11.59 12.76</td>
<td>0.39 0.57 0.0 0.03 0.78 0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A422</td>
<td>San Lucas, Baja</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A376</td>
<td>Rancho la Presa, Baja</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A429</td>
<td>Sierra Giganta, Baja</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A374</td>
<td>Todos Santos, Baja</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A572</td>
<td>Sil Nagya, Arizona</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A316b</td>
<td>Caborca, Sonora</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A514</td>
<td>Libertad, Sonora</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A361</td>
<td>El Desemboque, Sonora</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A512d</td>
<td>San Carlos, Sonora</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A300</td>
<td>Navojoa, Sonora</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative longevity than the females (1.60 and 1.96, respectively). When the response of the same sex is compared between substrates, males do not differ (1.60 and 1.59) but females from Arizona and Sonora live longer (1.96) than females from Baja (1.30). In fact, the difference between the response of females is responsible for the significant main effect of substrates. In addition, the significant main effect of ethanol is due to the 2.0% treatment showing the highest relative longevity (1.75) and the 4.0% treatment yielding the lowest response (1.51). One additional note is that the longest-lived female came from the 4.0% treatment vial of strain A514. She lived 881 hr (approximately 37 days) and yielded mature eggs on dissection.

Experiment 4. It is known that the (fast) product of the Adh-F allele of D. melanogaster is less stable to heat and pH changes than the (slow) product of the Adh-S form (3, 10). In-gel heat sensitivity tests were conducted with D. mojavensis by simultaneously immersing gel slices in a hot water bath (45°C). The slices were removed after 3, 7, and 11 min and stained for ADH activity. The results are given in Fig. 4. The activity of the Adh-F product was reduced after 7 min and almost lost after 11 min at 45°C. Acidity tests on enzyme activity were conducted with Tris-HCl staining buffers adjusted to pH 4.5, 6.0, and 8.5 with 0.1 M HCl. The reaction rate of Adh-F product was reduced at pH 6.0 and 4.5, but no difference in total activity was detected between the fast and slow forms.

DISCUSSION

Because many Drosophila species are associated with fermentative organisms, such as yeasts, it is expected that they should evolve systems to cope with the physiological stress imposed by the by-products of fermentation. There are four ways by which a species could cope with substrates containing alcohol. The first obvious method would be to avoid alcohol-rich substrates in preference for nonalcohol media. The exclusion of D. simulans from collections during vintage in the area of

Table 3. Analysis of variance for the effect of atmospheric ethanol on the relative longevity (measured as longevity of ethanol treatment/longevity of control) of strains from the two subraces of D. mojavensis

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subraces</td>
<td>1</td>
<td>1.002</td>
<td>14.59***</td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>0.062</td>
<td>0.91</td>
</tr>
<tr>
<td>Ethanol treatment</td>
<td>1</td>
<td>0.703</td>
<td>10.23**</td>
</tr>
<tr>
<td>Subraces x sex</td>
<td>1</td>
<td>0.935</td>
<td>13.61***</td>
</tr>
<tr>
<td>Subraces x ethanol</td>
<td>1</td>
<td>0.003</td>
<td>0.04</td>
</tr>
<tr>
<td>Sex x ethanol</td>
<td>1</td>
<td>0.001</td>
<td>0.01</td>
</tr>
<tr>
<td>Subraces x sex x ethanol</td>
<td>1</td>
<td>0.012</td>
<td>0.17</td>
</tr>
<tr>
<td>Residual</td>
<td>44</td>
<td>0.069</td>
<td></td>
</tr>
</tbody>
</table>

*α = 0.01.
***α = 0.001.

FIG. 2. Mean longevities in ln (hr) for axenic strains of D. mojavensis fed (i) sterile dead yeasts ●—●, (ii) Candida krusei ○—○, and (iii) K. apiculata X—X.
fermentation at an Australian wine cellar (1) demonstrates an avoidance strategy. Second, they could adapt enzyme systems that tolerate alcohol but do not utilize it as an energy source. Third, they could metabolize alcohol directly as an energy source, effectively detoxifying it. Finally, adults could benefit from the presence of microorganisms they have ingested, which metabolize alcohol in the digestive system of the fly.

The adaptation of enzyme systems for alcohol tolerance has been well documented for populations of *D. melanogaster*. McKenzie and Parsons (2) attempted to show that tolerance is independent of the ADH locus and that another polymorphism exists that determines the ability of strains to survive in alcohol-associated environments. Contrary to this opinion, Briscoe *et al.* (4) demonstrated that the ADH phenotype of *D. melanogaster* associated with wine cellars in Spain is an important component in the adults' ability to resist alcohol.

We have demonstrated an increase in longevity on exposure to alcohol vapors for the desert-adapted fly *D. mojavensis*. Our techniques for studying the effects of ethanol are less stringent on the organism than the methods used by the investigators of *D. melanogaster* and the longevity of this species is also increased by the present technique. In fact, Van Herreweghe and David (22) demonstrated an increase in longevity of *D. melanogaster* from 2 to 8 days under axenic conditions when 8% ethanol was added to a medium of agar. The increase in longevity of *D. mojavensis* is accompanied by the retention of mature eggs in females, suggesting a corresponding increase in fitness. That the increase in longevity is also independent of diet suggests an autonomous utilization of ethanol as was proposed for *D. melanogaster* (22).

The differential response of longevity of the subraces of the females is in the direction seemingly most favorable for survival. The longer-lived females from Arizona and Sonora breed in rotting organpipe cactus which is temporally and spatially less available than rotting agria cactus in Baja California. The major climatic differences between the regions occupied by the two subraces is that Sonora and southern Arizona have greater extremes in temperature and humidity than Baja California (23). This is reflected in the temperature of the substrates in the regions. Both organpipe and agria have mean rot temperatures around 27° but organpipe has a variance in substrate temper-
0.05%). The peak corresponding to isopropanol in the decaying tissues of organpipe had a concentration less than 0.01% in four samples tested. The fast ADH in D. melanogaster was found to have twice the activity of the slow ADH when isopropanol was used as the substrate (8). This is a possible positive selective agent for the fast ADH in populations of D. mojavensis. Thus, the environmental heterogeneity proposed for maintaining the alleles at the ADH locus is the interaction of substrate alcohol content with pH and temperature.

The frequency of Adh-F was negatively correlated with the response to alcohol treatments of 2.0 and 4.0% (r = -0.633 and -0.554, respectively). Even though the longevity response of subraces is correlated with the alleles at the ADH locus, more loci may be involved (2, 23). The ADH locus is located on the third chromosome of D. mojavensis (25); however, the frequency of the ST arrangement of the third chromosome was not found to be correlated with the response to strains to ethanol treatments of 2.0 and 4.0% (r = -0.131 and 0.004, respectively). The frequency of the LP arrangement on the second chromosome was found to be correlated with the response to ethanol treatments of 2.0 and 4.0% (r = -0.694 and -0.713, respectively). The octanol dehydrogenase locus is located on the 2nd chromosome (25) and could be responsible for this observed correlation. The second chromosome could contain modifier or regulator genes that respond to environmental ethanol.

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