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ETHANOL UTILIZATION AND TOLERANCE IN LABORATORY POPULATIONS OF *Drosophila melanogaster*

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ABSTRACT

Due to limitations and problems with calculation of the ethanol concentration that kills 50% of the individuals after ethanol exposure (LC50), nonparametric statistical methods were used for the analysis of survival data of three laboratory populations of *Drosophila melanogaster*. These populations were collected seven years ago in a cellar, a vineyard and a pinewood.

There was a positive correlation between the beneficial effect of ethanol at low concentrations and the toxic effect at high concentrations among the three populations and between the two sexes within each population, although the effects of sucrose were also important. Survival and alcohol tolerance varied among the three populations.

The pattern of response of both sexes together was different from male and female patterns analysed separately. This is indicative that the sexes should not be grouped for data analysis without determining whether they have the same response.

INTRODUCTION

Ethanol is a primary alcohol which is made naturally by the fermentation process carried out by yeasts. Ethanol is an ecological factor of great importance for *Drosophila melanogaster*, since at low concentrations the species uses ethanol as an energy resource (van Herrewege and David, 1974; Deltombe-Lietaert *et al.*, 1979; Anderson *et al.*, 1981); whereas at high concentrations it is toxic (David and Bocquet, 1974; Kerver and van Delden, 1985).

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To compare ethanol tolerance among similar species, different studies in natural populations have been made (David *et al.*, 1974, 1979), mainly between *D. melanogaster* and *D. simulans* (McKenzie and Parsons, 1972; David and Bocquet, 1976; David *et al.*, 1977). Both of these sibling species, can use ethanol, at low concentrations, as a nutritive resource in nature, but when the ethanol level is high the two species occupy different niches. This fact explains the absence of *D. simulans* inside cellars, whereas *D. melanogaster* is the most abundant species (Monclús and Prevosti, 1978).

In studies about tolerance, comparing *D. melanogaster* populations captured inside cellars and at different distances from cellars, the highest tolerance was found in the cellar and a cline according to the distance from the cellar was observed (McKenzie and Parsons, 1974; Hickey and McLean, 1980).

The existence of natural selection operating in habitats associated with ethanol in nature and the capacity of *D. melanogaster* to adapt to those conditions is supported by studies in laboratory populations (David and Bocquet, 1977; Gibson *et al.*, 1979; Dorado and Barbancho, 1984; Oakesshott *et al.*, 1985).

Van Herrewege and David (1980) found a weak correlation between ethanol tolerance and utilization, since an increase of tolerance made by selection is not always correlated with more efficient use. Thus, these two physiological traits are controlled, at least partially, by different genetic mechanisms.

Because of the lack of works comparing ethanol utilization inside and outside cellar populations, three laboratory populations coming from a cellar, a vineyard, and a pinewood have been studied. It was assumed that the cline from higher to lower tolerance would be CELLAR > VINEYARD > PINEWOOD as other authors have found (McKenzie and Parsons, 1974; Hickey and McLean, 1980).

On the other hand, because of limitations and problems to calculate the ethanol concentration that kills 50% of the individuals after a determined time of ethanol exposure (LC50) in *D. melanogaster*, nonparametric statistical methods for survival data analysis were introduced.

MATERIALS AND METHODS

D. melanogaster strains

The experiment was carried out with 22 isofemale strains coming from each of three populations captured in three different places: a wine cellar in Requena (Valencia), a vineyard in El Pontón, 4 km away from the cellar, and a pinewood at la Cañada (Valencia) located 70 km away from Requena. The strains have been maintained for seven years in laboratory conditions at 19 ± 1°C.

Adult survival test

The protocols of David *et al.* (1974) and McDonald *et al.* (1977) were followed, with some small modifications: In 10 x 2.5 cm vials, ten 4-5 day old individuals were placed. Two sexes, seven ethanol concentrations (0, 2, 5, 7, 10, 12, and 15% ethanol) and twenty-two strains of each one of the three populations were tested. Two replicates for each sex were made.

To each vial five strips of filter paper (5 x 2.5 cm) saturated with 1 ml of a water solution of 0.3% sucrose with 0, 2, 5, 7, 10, 12, or 15% ethanol were added. Vials were hermetically closed with parafilm to prevent evaporation of alcohol. Flies were maintained at 25 ± 1°C with constant light. The counts of dead flies were carried out at 5, 10, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, and 288 hours.

Measure of oxygen consumption

Conventional manometrics methods (Umbreitt *et al.*, 1972) were used to measure the oxygen consumption of flies at rest. A Gilson's differential constant-pressure respirometer was used. In each flask covered with a gauze there were 50 flies and 0.5 ml of 20% KOH and a piece of 2 x 3 cm filter paper were placed inside.

The measure of oxygen consumption was made one hour and 15 min after the flasks were attached to the branches of the respirometer.

Statistical methods

The Mantel-Haenszel's chi-square test to compare the survival curves in pairs (Lee, 1980; Kalbfleish and Prentice, 1980) and the Lee-Desu's test to compare the survival curves together and in pairs (available in the SPSS statistical package) were used.

After applying the Mantel-Haenszel's chi-square test and the Lee-Desu's test it was seen that the first is more conservative than the second, since it requires a greater difference from the null hypothesis to reject the equality of the distributions. For this reason, only the results from the Lee-Desu's test will be shown.

Ethanol use at low concentrations and toxic effects at high concentrations are clear from the survival curves for each concentration, when compared to 0% concentration. When a survival curve is above the control survival curve (0% ethanol), this indicates ethanol use. When a survival curve is below the control survival curve a toxic effect of ethanol is indicated.

In order to compare the toxic effects of ethanol the median of the survival time distribution was used.

To compare the use of ethanol the median was used in the first instance. For those cases in which more than 50% of individuals survived when the experiment was completed the first quartile of the distribution was considered for comparison. The percentile of order 10 was considered when more than 75% of individuals survived at the end of the experiment.

RESULTS

In Figures 1, 2 and 3 it can be observed that at 2 to 7% ethanol, males and females of the three populations (cellar, vineyard and pinewood), had a higher survival than at 0%. On the other hand, survival at 10 to 15% ethanol was always lower than at 0%.

Table I shows that for males the survival curves at 0% and 7% are not significantly different ($P < 0.05$). But in females the two survival curves (0% and 7%) are significantly different. It can be seen though, that the threshold between beneficial and toxic effect of ethanol is close to 7% in males, but it is higher in females, although not reaching 10% ethanol.

In Table II the order of survival among the three populations is shown according to the Lee-Desu's test and the observation of the survival curves. From 0% up to 7%, PINEWOOD and VINEYARD populations have a survival significantly higher than the CELLAR population; nevertheless, from 10% concentration onwards, CELLAR and VINEYARD populations have a survival significantly higher than the PINEWOOD population, except at 10% concentration in males and at 15% in females where VINEYARD and PINEWOOD do not have significantly different survival curves. CELLAR and VINEYARD populations have a pattern in males (CELLAR = VINEYARD) different from the pattern in females: at 10% VINEYARD > CELLAR and at 12% and 15% CELLAR > VINEYARD.

The results obtained by contrasting survival curves by means of the Lee-Desu's test show a positive correlation between the beneficial effect of ethanol at low concentrations and the toxic effect at high concentrations among the three populations and between both sexes within each population. The population or sex that has greatest beneficial effect of ethanol is also the population or sex that suffers the greatest toxic effect.

Calculation of the percentage of relative mortality against the ethanol concentration in the three populations studied and in the two sexes for each population (Table III) confirms the positive correlation between both effects of ethanol, decreasing the possibility that this correlation could be due to a sucrose effect and not to an ethanol effect.

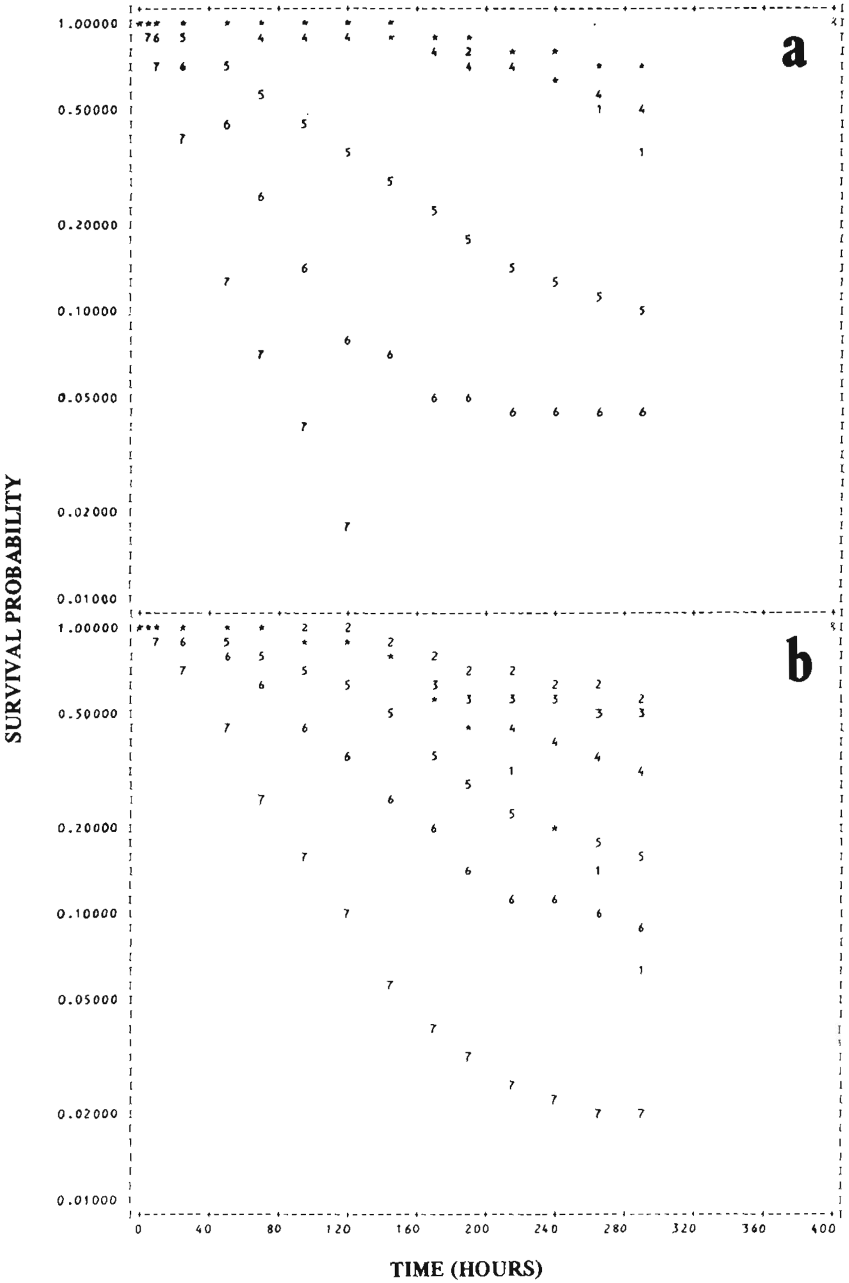

Figure 1 - Survival curves at each of 7 ethanol concentrations for males (a) and females (b) of the CELLAR population. The survival curves are designated by 1 for 0%, 2 for 2%, 3 for 5%, 4 for 7%, 5 for 10%, 6 for 12% and 7 for the 15% ethanol concentration. Asterisks indicate that survival curves coincide at these points.

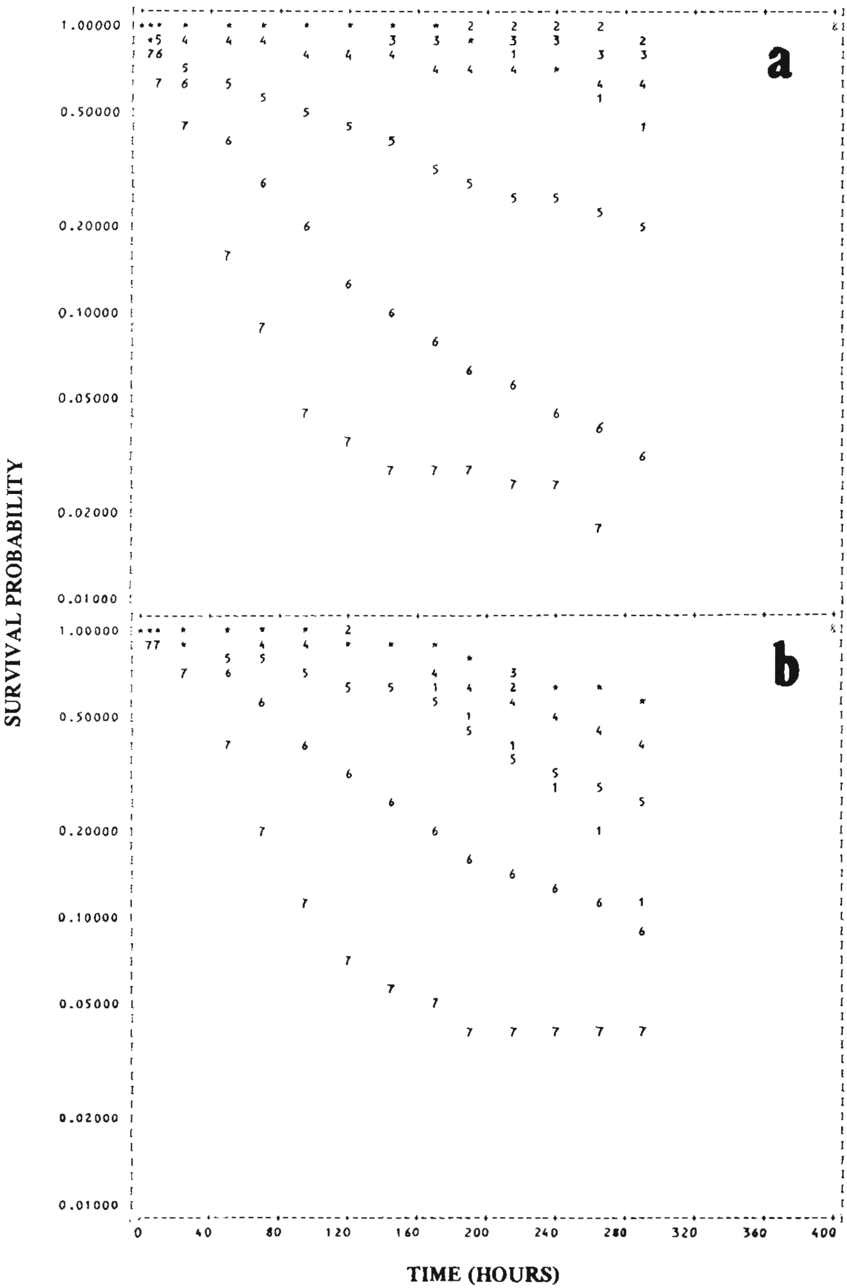

Figure 2 - Survival curves for each of 7 ethanol concentrations for males (a) and females (b) of the VINEYARD population. See Figure 1. Legend.

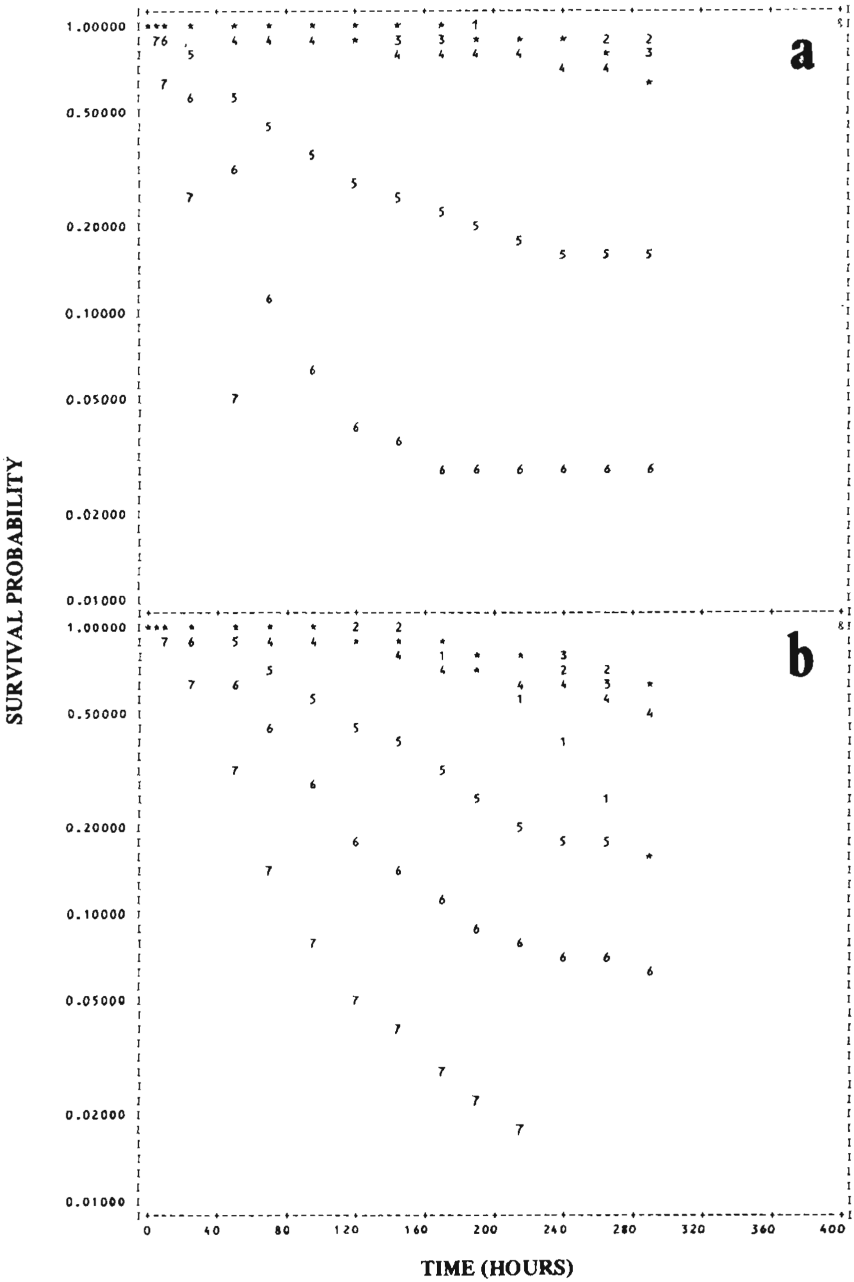

Figure 3 - Survival curves for each of the 7 ethanol concentrations for males (a) and females (b) in the PINEWOOD population. See Figure 1. Legend.

Table I - Significances of the Lee-Desu's statistic values (probability of obtaining a similar or a higher statistic value) obtained by comparing in pairs the survival curves of each sex at each concentration and population. The order followed is:

0% - 2%	0% - 5%	0% - 7%	0% - 10%	0% - 12%	0% - 15%
2% - 5%	2% - 7%	2% - 10%	2% - 12%	2% - 15%	
5% - 7%	5% - 10%	5% - 12%	5% - 15%		
7% - 10%	7% - 12%	7% - 15%			
10% - 12%	10% - 15%				
12% - 15%					

	Males						Females					
Cellar	0	0	0.513	0	0	0	0	0	0.001	0	0	0
	0.988	0	0	0	0		0	0	0	0	0	
	0	0	0	0			0	0	0	0		
	0	0	0				0	0	0			
	0	0					0	0				
	0						0					
Vineyard	0	0	0.377	0	0	0	0	0	0	0.001	0	0
	0	0	0	0	0		0.678	0	0	0	0	
	0	0	0	0			0	0	0	0		
	0	0	0				0	0	0			
	0	0					0	0				
	0						0					
Pinewood	0	0	0.081	0	0	0	0	0	0	0	0	0
	0.024	0	0	0	0		0.859	0	0	0	0	
	0	0	0	0			0	0	0	0		
	0	0	0				0	0	0			
	0	0					0	0				
	0						0					

Table II - In the upper part of each cell the significances of the Lee-Desu's statistic values are shown. These significances have been obtained by comparing in pairs the survival curves in the order: CELLAR-VINEYARD, CELLAR-PINEWOOD and VINEYARD-PINEWOOD. Below the significance values the order from higher to lower survival obtained by considering the survival curves together with the Lee-Desu's statistic value is shown. $P \leq 0.05$.

<table>
<tr><th></th><th colspan="3">Males</th><th colspan="3">Females</th></tr>
<tr><td>0%</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td></td><td colspan="3">Pinewood > Vineyard > Cellar</td><td colspan="3">Pinewood > Vineyard > Cellar</td></tr>
<tr><td>2%</td><td>0</td><td>0</td><td>0.003</td><td>0.830</td><td>0.061</td><td>0.069</td></tr>
<tr><td></td><td colspan="3">Vineyard > Pinewood > Cellar</td><td colspan="3">Vineyard = Pinewood = Cellar</td></tr>
<tr><td>5%</td><td>0.040</td><td>0.011</td><td>0.549</td><td>0</td><td>0</td><td>0.204</td></tr>
<tr><td></td><td colspan="3">Vineyard = Pinewood > Cellar</td><td colspan="3">Vineyard = Pinewood > Cellar</td></tr>
<tr><td>7%</td><td>0.186</td><td>0.004</td><td>0.311</td><td>0</td><td>0</td><td>0.027</td></tr>
<tr><td></td><td colspan="3">Vineyard
|| ||
Pinewood > Cellar</td><td colspan="3">Pinewood > Vineyard > Cellar</td></tr>
<tr><td>10%</td><td>0.949</td><td>0.006</td><td>0.159</td><td>0.002</td><td>0.009</td><td>0</td></tr>
<tr><td></td><td colspan="3">Vineyard
|| ||
Cellar > Pinewood</td><td colspan="3">Vineyard > Cellar > Pinewood</td></tr>
<tr><td>12%</td><td>0.179</td><td>0</td><td>0.026</td><td>0.021</td><td>0</td><td>0.005</td></tr>
<tr><td></td><td colspan="3">Cellar = Vineyard > Pinewood</td><td colspan="3">Cellar > Vineyard > Pinewood</td></tr>
<tr><td>15%</td><td>0.469</td><td>0</td><td>0.003</td><td>0.011</td><td>0</td><td>0.153</td></tr>
<tr><td></td><td colspan="3">Cellar = Vineyard > Pinewood</td><td colspan="3">Cellar > Vineyard = Pinewood</td></tr>
</table>

With respect to oxygen consumption, Table IV shows the means and standard errors of males and females of the three populations. The similarity of the means was confirmed by two way ANOVA, which showed no significant differences among populations nor between sexes.

Table III - In the upper part of each cell the significances of the Lee-Desu's statistic values are shown. These significances have been obtained by comparing the survival curves of males and females at each concentration and population. Below the significance values the order from higher to lower survival obtained by considering the survival curves together with the Lee-Desu's statistic value is shown. $P \leq$.

	Cellar	Vineyard	Pinewood
0%	0 Males > Females	0 Males > Females	0 Males > Females
2%	0 Males > Females	0 Males > Females	0 Males > Females
5%	0 Males > Females	0 Males > Females	0 Males > Females
7%	0 Males > Females	0 Males < Females	0 Males > Females
10%	0 Males < Females	0 Males < Females	0 Males < Females
12%	0 Males < Females	0 Males < Females	0 Males < Females
15%	0 Males < Females	0 Males < Females	0 Males < Females

DISCUSSION

The LC50 determination has limitations: a) Its calculation is confined to toxic ethanol concentrations. b) The time interval in which it is possible to apply the LC50 depends on the species and the toxic agents used. c) In vials closed airtightly at toxic concentrations nearly all the flies died because of ethanol ingestion, ethanol assimilation through the respiration or progressive anoxia; therefore, the LC50 can be correctly calculated only during the first hours.

Table IV - Number of replicates and means with their standard errors of the metabolic rates expressed in μl $O_2.mg^{-1}$ fresh weight.hour^{-1} of males (M) and females (F) of the three analysed populations.

Population	Replicates		Mean ± Standard error	
			μl $O_2.mg^{-1}$ fresh	weight hour^{-1}
	M	F	M	F
Cellar	21	22	3.69 ± 0.25	3.33 ± 0.27
Vineyard	23	21	3.43 ± 0.17	3.44 ± 0.21
Pinewood	18	23	3.66 ± 0.31	3.10 ± 0.18

For these reasons non-parametric tests, to compare survival curves, have been carried out in this work. Non parametric statistics, provide a more efficient, clear and precise method to distinguish tolerances to a toxic agent such as ethanol among different populations.

The survival order when sucrose is the only energetic resource is pinewood > vineyard > cellar. It is hardly surprising because there is more sugar in the cellar than in vineyards and in pinewoods. Therefore, the survival order could be explained by a previous adaptation to the environment of each population: A population in a habitat with sugar scarcity must adapt itself to these conditions by using this sugar more effectively than a population with sugar abundance.

On the other hand, survival at 0% ethanol concentration is higher in males than in females. This fact could be due to the size of the individuals since males are smaller than females and, for this reason, they have more sucrose, and/or oxygen/unit body wt. than females within tightly closed vials.

In general, at a low ethanol concentration the order of survival is pinewood > vineyard > cellar and at high ethanol concentrations, the order of survival is inverted (cellar > vineyard > pinewood). Therefore, the population that better uses the ethanol also suffers more from toxic effects.

Other studied indirectly show this correlation between both alcohol effects. For example, van Herrewege *et al.* (1980) studying the effect of three primary alcohols (ethanol, propanol and butanol) in a strain homozygous for the F allele of the Adh locus found that butanol was the alcohol best used, although it was also the most toxic one. Anderson *et al.* (1981) studying two isogenic strains, S-1 and F-1, homozygous for the second, third and X chromosomes, found that at a low ethanol concentration (0.5%), the

S-1 strain had a higher LT50 than the F-1 strain but at high ethanol concentrations (6, 8 and 10%) the F-1 strain survived significantly longer than the S-1 strain.

From the van Herrewege and David studies (1980, 1984) with *D. melanogaster* populations from different latitudinal origins, it can be deduced that before artificial selection, the population from a temperate climate uses ethanol as a nutritional resource less efficiently than the tropical populations, although the former are more tolerant to the toxic ethanol concentrations.

To explain the tolerance cline (cellar > vineyard > pinewood) in our populations a partial genetic independence between the ethanol tolerance and the capacity to use it as a nutritive resource could be assumed (van Herrewege and David, 1980, 1984; Dorado and Barbacho, 1984). It is possible that inside the cellar, selection acts by improving the ethanol tolerance because of the high ethanol concentration in this habitat while in the pinewood population selection must improve the ethanol utilization due to the scarcity of alcohol in relation to the cellar. The vineyard population is subjects to environmental conditions halfway between the other two populations; so, its behavior upon selection is also halfway.

David and Bocquet (1977) maintained for two years a French strain in laboratory conditions without changes in the ethanol tolerance. In our populations, which are seven years old, since the cline of tolerance remains, it is reasonable to postulate that ethanol tolerance is controlled by a polygenic system. Other studies (Cavener and Clegg, 1981; Middleton and Kacser, 1983) give evidence of this polygenic determination.

The same positive correlation between the use of ethanol and its toxic effect can be observed between sexes in each population. So, males better use ethanol at low concentrations but are less tolerant to high concentrations. This fact shows that sexes should not be grouped for data analysis without determining if the response is the same.

The lower tolerance of males could be explained by the effect that the ethanol fumes exert on the flies within tightly closed vials. The different surface/volume relation between males and females could be important in explaining this fact: males must incorporate relatively more ethanol fumes through their trachea than females; so, at the same metabolic rate, expressed in µl O_2/mg fresh weight/hour, they would be less tolerant than females.

Other studies (David and Bocquet, 1974, 1976; David *et al.*, 1974; Kerver and van Delden, 1985) show a higher tolerance in the females although Sanchez-Cañete *et al.* (1986) found that females from a winery were less tolerant to ethanol than males.

With respect to the ethanol utilization there is a controversy; while Sanchez-Cañete *et al.* (1986) found the best use of ethanol among males in their control lines, Ziolo and Parsons (1982) found that females better used ethanol fumes in isofemale strains from a winery. Van Herrewege and David (1984) carried out a selection to improve the ethanol utilization and found that the best use of ethanol for either sex depends on the generation and that there are no significant differences.

Therefore, the positive correlation found between the use of ethanol as a nutritive resource and its toxic effect among the populations as well as between the sexes could be due to the differences in use of either sucrose or ethanol.

The estimate of the relative mortality seems to corroborate that this correlation is due to the ethanol effect and not to the sucrose. Nevertheless, other studies will be necessary to clearly distinguish sucrose and ethanol effects and to confirm this suggestive hypothesis: greater use of ethanol means a higher toxic effect.

RESUMO

Devido as limitações e problemas para calcular a concentração tóxica do etanol que mata 50% dos indivíduos após um determinado período de exposição (LC50), métodos estatísticos não-paramétricos foram usados para analisar os dados de sobrevivência de três populações de laboratório de *Drosophila melanogaster*. Estas populações foram coletadas a sete anos atrás em um celeiro, em um vinhedo e em um pinheiral.

Os resultados mostraram uma correlação positiva entre os efeitos benéficos do etanol a baixas concentrações e o efeito tóxico a altas concentrações entre as três populações e entre os dois sexos em cada população, entretanto os efeitos da sacarose também foram importantes. A sobrevivência e a tolerância ao álcool variaram entre as três populações.

O padrão de resposta dos sexos juntos foi diferente dos padrões de machos e fêmeas analisados separadamente. Isto indica que os sexos não devem ser agrupados para os dados analisados sem determinar se ambos tem a mesma resposta.

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