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Ethanol tolerances of *Drosophila melanogaster* populations selected on different concentrations of ethanol supplemented media

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Summary. Eight recently collected Australasian populations of *D. melanogaster* were each divided into eight selection lines. Two of these lines from each population were maintained on one of four types of selection media: standard food supplemented with 0%, 3%, 6% and 9% ethanol. After 30 generations the selection lines were tested for tolerance to 9% ethanol medium and after another 20 generations adults were tested for tolerance to concentrated ethanol fumes. Significant differences in tolerance were found among lines selected on different media which were consistent across the eight populations. On the 9% test media, the 6% and 9% selection lines, as compared with the control lines selected on 0% ethanol, were more likely to survive as pre-adults or adults, faster to develop as preadults, and heavier and more productive as adults. However, the tolerance of the 3% lines to the 9% test media was less than that of the 0% control lines in preadult and adult survival, intermediate between that of the 0% and the 6% and 9% lines in productivities, and apparently superior to the 6% and 9% lines in development times and adult weights. The 3%, 6% and 9% lines showed similar tolerances to the ethanol vapour. Previous work showed that 3% ethanol can be a metabolic benefit to *D. melanogaster* but 6% and 9% are metabolic costs. The present results suggest that the phenotype selected on 3% to obtain a metabolic benefit differs in many respects from that selected on 6% and 9% to minimise their detrimental effects.

Key words: *Drosophila* – Selection – Ethanol tolerance – Alcohol dehydrogenase

Introduction

It has long been an elusive goal of population genetics to establish the relationships between genetic polymorphisms, adaptively significant phenotypes and environmental variation. One model system which has been extensively studied in *Drosophila melanogaster* is the polymorphism for the enzyme alcohol dehydrogenase (*Adh*), the phenotype of ethanol tolerance and the level of ethanol in the feeding environment (van Delden 1982; Gibson and Oakeshott 1982). Ethanol is a component of many substrates on which wild *D. melanogaster* feed, reaching levels around 3% in fermenting fruit and above 12% in fresh wine seepages in winery cellars (Gibson et al. 1981; Oakeshott et al. 1982). Further, the three polymorphic allozymes of *Adh* differ substantially in their in vitro activities for ethanol as substrate (Gibson and Oakeshott 1982).

However, qualitatively different responses of *Adh* allele frequencies have been obtained in different selection experiments in ethanol environments (see Gibson and Oakeshott 1982 for further discussion). Methodological differences between the previous studies which may explain the different selection responses include the method of administering the alcohol (supplemented to the food medium or as a vapour mixture in air), the fitness component measured (e.g. development time, survival, longevity) and the type of population used (inbred versus outbred, laboratory adapted versus wild-caught). One particularly important methodological difference may be the concentration of ethanol administered, which has varied from 1% to 30%. van Herrewege and David (1980) and Parsons and Stanley (1981) have suggested that while relatively high concentrations of ethanol are undoubtedly detrimental, low concentrations can be advantageous to the flies as an energy source. van Herrewege and David (1980) have some evidence that tolerance to high concentrations and utilisation of lower doses may involve at least partly different physiological processes.

Accordingly, we have carried out a selection experiment for adaptation to various levels of ethanol in the food, ranging from 0%, through the putatively advantageous 3%, up to the stressfully high concentrations of 6% and 9%. *Adh* allele frequency changes during the course of the experiment will be published elsewhere (Oakeshott et al. 1984). Here we report the tolerance of the lines selected on the four concentrations to a test dose of 9% ethanol in the food. Tolerance to this test dose is assessed in several components of fitness. In order to check for cross-tolerance to gaseous ethanol, we also report the knockdown resistance (Cohan and Graf 1985) of the selected lines to stressful levels of ethanol vapour mixed in air.

Materials and methods

The experiment involved eight base populations each established from a single collection of 20 fertilised females. The eight collection sites were Sogeri (Papua New Guinea, 8.8°S, 144.3°E), Darwin (Northern Territory, 12.5°S, 130.8°E), Palm Woods (Queensland, 26.6°S, 153.0°E), Brisbane (Queensland, 27.5°S, 153.0°E), Saint Peters (South Australia, 34.9°S, 138.6°E), Coriole (South Australia, 35.0°S, 138.5°E), Melbourne (Victoria, 37.7°S, 144.8°E) and Cygnet (Tasmania, 43.0°S, 147.3°E) (details in Oakeshott et al. 1984). Anderson (1979, 1982) showed that the ethanol tolerance of the base populations as assessed from adult survival on 15% ethanol supplemented food increased with increasing distance from the equator.

After between four and seven generations in the laboratory eight random samples of 80 flies were taken from each base population to establish eight selection lines. Two of these eight selection lines were transferred to each of four different types of media: standard medium supplemented with 0%, 3%, 6% or 9% (v/v) ethanol (the food was cooled to between 48° and 55 °C before the ethanol was added; recipe in Oakeshott and Gibson 1981). The total of 64 selection lines were maintained on their respective media types for 30 discrete four-week generations at 22 ± 2 °C. For each selection line about 40 males and 40 females were transferred each generation.

In order to assess the response after 30 generations of selection, several fitness components were measured on subcultures of the 64 selection lines on medium supplemented with 0% (as a control) and 9% ethanol. All subcultures tested were cultured for one generation on 0% ethanol medium prior to exposure to the test dose. *Egg-to-adult survival* was measured with eggs laid on 0% medium over a 12 h period which were then transferred in cohorts of 20 to the test medium. One such cohort was set up for each selection line on each of the 0% and 9% ethanol test media. *Adult survival* was measured on single-sex cohorts of 20 flies which were aged 4–6 days since emergence on 0% ethanol medium prior to the test. They were then transferred to the test medium and three days later the survivors were counted and removed. Generally two cohorts of each sex were tested for each selection line on each of the 0% and 9% test media. The progeny of the female cohorts on the test media then provided estimates of three other fitness components. *Larval development time* was measured as the number of days until the first 15 progeny in each culture had pupated. *Productivity* was measured as the total number of adult progeny alive two weeks after the emergence of the first 15 adults. At this time *Adult weight* was measured for each sex by weighing a random sample of 20 flies.

After 50 generations of selection the *Knockdown resistance* of adults was measured in air nearly saturated with ethanol fumes. It was not feasible to test all selection lines under this treatment so only the lines from four base populations representing the collections from the latitudinal extremes were examined. All subcultures tested were cultured for a generation on 0% ethanol before the test. The adults tested were aged 2–6 days since emergence. The apparatus and protocol for measuring knockdown resistance are described in detail in Cohan and Graf (1985). Briefly, a mixed-sex cohort of about 200 adults was shaken without anesthesia into a long, vertical glass tube through which air nearly saturated with ethanol fumes was pumped. As flies succumbed to the fumes and could no longer keep their posture, they would roll down a series of baffles to the bottom of the tube. The purpose of the baffles was to ensure that a fly that had only partially succumbed had numerous opportunities to stop itself from rolling to the bottom. When an individual did roll to the bottom it would land on a collecting dish, which was replaced every minute. The flies that fell each minute were separated by sex and counted, yielding the entire distribution of knockdown times of each sex.

In respect of the power of the analyses below, it should be noted that the total numbers of cohorts tested for knockdown resistance and egg-to-adult survival were only about a quarter and a half, respectively, of the number for the other fitness components.

Results

Ultimately the aim of the experiment was to compare the response of each fitness component in the lines selected on the three types of ethanol-supplemented media to that in the control lines maintained on medium with no added ethanol. However, the most appropriate way to make this comparison depended on the outcome of preliminary analyses. These were two-factor analyses of variance evaluating the heterogeneity in response due to the three ethanol selection concentrations (3%, 6% and 9%), the eight base populations, their interaction and the two replicate lines within each concentration-population combination (Table 1).

In these preliminary analyses the main effect due to base populations was only significant for a minority of the fitness components measured and no fitness component in any test environment was significantly affected by the interaction between base populations and selection concentrations. However, the main effect of selection concentration was significant for several fitness components, particularly on the 9% test media. Thus, the three ethanol selection concentrations gave homogeneous results for knockdown times in gaseous ethanol and the only component in which they gave significantly different results on the 0% test medium was larval development time. On the other hand, on the 9% test medium, significant divergence among the three selection concentrations was found for all fitness components except adult female survival, and even for the latter the differences approached significance ($0.05 < P < 0.10$).

Table 1. Two-way analyses of variance for the effects due to the three ethanol concentrations in the selection medium (3%, 6% and 9%) and the eight base populations on all fitness components measured

Fitness component[a]	Percentage of variance due to			
	Selection conc. (E)	Base population (P)	E×P	Replicate lines
Knockdown times in gaseous ethanol				
adult males	−6	22*	17	67
adult females	−8	14*	41*	54
Survival on 9% ethanol medium[b]				
egg-to-adult	37***	15	17	32
adult males	16****	2	43*	38
adult females	5*	−7	2	99
Other components on 0% test medium				
development time	8**	−2	29	66
productivity	0	25***	57*	17
adult male weight	0	12	22	67
adult female weight	−1	5	36	59
Other components on 9% test medium				
development time	44****	−16	−11	83
productivity	7***	18**	55*	20
adult male weight	4**	24**	49*	23
adult female weight	9**	46***	14	32

* $P<0.25$, ** $P<0.05$, *** $P<0.01$, **** $P<0.001$

[a] Survival data were angularly transformed, development times and weights were logarithmically transformed and productivities square root transformed prior to analysis

[b] Results for survival on 0% ethanol test medium are not tabulated since, as expected, mortality rates on this medium were consistently small

The following strategy was adopted to compare results from lines selected on the ethanol supplemented media with those from the 0% selection medium. If the three ethanol selection concentrations gave homogeneous results for a particular fitness component (Table 1), they were pooled for comparison with the 0% selection lines. If on the other hand they were too heterogeneous to warrant pooling ($P<0.25$, Sokal and Rohlf 1969), the three ethanol selection concentrations were compared separately to the 0% selection lines. Likewise, if the eight base populations gave homogeneous results and there was no base population-selection concentration interaction (Table 1), then the results for all base populations were pooled in an unpaired *t*-test comparing lines selected on ethanol supplemented media with those selected on 0% medium. However, if either the base population or interaction effects were sufficient not to warrant pooling across base populations ($P<0.25$), then a paired *t*-test was carried out; thus the difference between the mean of the ethanol selected lines and the 0% selection lines was calculated for each base population and then the mean of this difference across all base populations was tested against zero in a *t*-test.

In Table 2 the above criteria are applied to the results for knockdown times in gaseous ethanol and survival percentages on 9% test media. For both sexes, knockdown times of lines selected on the three ethanol concentrations were significantly longer than those of control lines selected on 0% medium. For all three measures of survival on the 9% test medium, the 6% and 9% selection lines showed higher levels of survival than did the 0% selection lines (although for only three of the six comparisons were the differences significant). However, the 3% selection lines were similar to the 0% lines in the survival of adult females and were significantly lower than the 0% lines in egg-to-adult and adult male survival percentages. Thus, if it is assumed that ethanol tolerance is represented by longer knockdown times and higher survival percentages, then the 6% and 9% selection lines were more tolerant than the 0% controls. Although the 3% lines were more tolerant than controls in knockdown times, they were no more tolerant than controls, and in some cases less so, in survival.

Table 3 summarises the results for development time, productivity and adult weight on the 0% and 9% test media. Firstly, considering the results from the 0% test medium, development was faster, productivity higher and adult female weight greater in the control lines selected on medium without added ethanol than in the lines selected on the three ethanol concentra-

Table 2. Means and standard errors of knockdown times in gaseous ethanol and survival percentages on 9% ethanol medium for the 0%, 3%, 6% and 9% lines. Combined estimates are shown when the 3%, 6% and 9% lines gave homogeneous results (Table 1). Significant differences between the lines selected on the ethanol supplemented and 0% ethanol media are also indicated

Fitness component	Selection medium				
	0%	3%	6%	9%	3%, 6%, 9% combined
Knockdown times in gaseous ethanol					
adult males (min)	14±1	17±2	16±1	18±1**	17±1**
adult females (min)	17±1	21±3	18±2	21±2	20±1**
Survival on 9% ethanol medium					
egg-to-adult (%)	51±7	34±6**	59±8	69±6**	–
adult males (%)	93±1	89±2***	95±1	95±1	–
adult females (%)	85±2	86±2	89±2**	90±2**	–

** $P<0.05$, *** $P<0.01$

Table 3. Means and standard errors of larval development times, productivities and adult weights on 0% and 9% ethanol test media for the 0%, 3%, 6% and 9% selection lines. Combined estimates are shown when the 3%, 6% and 9% lines gave homogeneous results (Table 1). Significant differences between the ethanol supplemented and 0% selection lines are also indicated

Fitness component[a]	Selection medium				
	0%	3%	6%	9%	3%, 6%, 9% combined
On 0% test medium					
development time	10.1± 0.2	10.4± 0.2	10.8± 0.1****	11.0± 0.1****	–
productivity	269 ±11	215 ±29**	180 ±13***	201 ±13****	199±12****
adult male weight	79 ± 2	78 ± 2	80 ± 2	81 ± 2	80± 1
adult female weight	118 ± 3	109 ± 3****	114 ± 2	111 ± 3*	111± 2***
On 9% test medium					
development time	16.0± 0.8	13.3±0.3****	14.8± 0.3**	15.1± 0.4	–
productivity	38 ±	61 ± 7**	75 ±10***	87 ± 9****	–
adult male weight	67 ± 2	80 ± 1***	75 ± 2**	76 ± 3	–
adult female weight	95 ± 4	110 ± 1***	102 ± 2	104 ± 5	–

** $P<0.05$, *** $P<0.01$, **** $P<0.001$

[a] Units are days for development time, numbers of flies for productivity and mg × 10^2 for weights

tions. Development time became progressively slower in lines selected on higher ethanol concentrations but productivities and adult female weights were homogeneous across the three ethanol selection concentrations. There were no significant differences in adult male weights either among the three ethanol selection concentrations or between these and the control lines. Presuming that faster development, higher productivity and greater weight represent greater fitness, then the general result (adult male weights excepted) is that the response to selection on ethanol supplemented medium was at the expense of fitness on unsupplemented medium.

Turning to the results in Table 3 from the 9% test medium, the 3%, 6% and 9% selection lines all showed greater productivities, greater adult weights and faster development times than the 0% controls. But for all three components of fitness there was heterogeneity among the three selection concentrations in the extent of the difference from the controls. The lines selected on the higher ethanol concentrations (6% and 9%) showed greater increases in productivity than the 3% lines. However, the 3% selection lines showed faster development and greater adult weights than the 6% and 9% lines.

Discussion

The general result across all fitness components tested is that maintaining flies for 30 generations on 6% or 9% ethanol media selects flies more tolerant to 9% ethanol medium than does maintaining them on 0%. This indicates that there was considerable genetic variation on which selection for tolerance to stressful ethanol levels could operate within each of the eight wild-caught base populations. The results corroborate those of David et al. (1977) and Gibson et al. (1979), who were also

able to select for increased survival to stressful ethanol levels in base populations recently collected from the wild.

Since the present study assessed the response to selection in several fitness components, it is possible to describe at least in part the tolerance phenotype being selected on stressful levels of ethanol in the medium. Thus, when tested on 9% ethanol after the thirty generations, the 6% and 9% selection lines were more likely than the 0% lines to survive as preadults or adults, faster to develop as preadults, and heavier and more productive as adults.

One particularly interesting aspect of the tolerance phenotype selected in the 6% and 9% selection lines was their increased resistance to stressful levels of ethanol vapour. This may reflect some shared physiological mechanisms of coping with gaseous ethanol and ethanol in the food (despite some opposite effects on *Adh* allele frequencies – see below). Alternatively, it is also possible that the mechanisms are different, at least in part, and that the resistance to gaseous ethanol developed in parallel to that for food ethanol because selection lines were consistently exposed to gaseous ethanol evaporating from the media. It has been shown that ethanol medium initially prepared to 9% contains only 3% after 10 days at 22 ± 2 °C (Oakeshott et al. 1983).

While the results from the 6% and 9% lines provide consistent and conclusive evidence for the selection of a tolerance phenotype on stressful ethanol levels in the medium, the results for the 3% lines reveal a very complex pattern of adaptation. When tested on 9% ethanol the tolerance of the 3% lines was less than that of the 0% lines in egg-to-adult and adult survival, intermediate between that of the 0% and the 6% and 9% lines in productivities, and apparently superior even to those of the 6% and 9% lines in development times and adult weights. When tested with the gaseous ethanol the 3% lines showed similar tolerance to the 6% and 9% lines.

In fact the apparent superiority of the 3% lines in development time and adult weight on the 9% test dose may be an artifact due to their very low survival rates on this dose. Lower larval survival will lead to lower larval densities and the latter could in turn allow the output of heavier and more rapidly developing flies. Furthermore, if, as seems reasonable, it is the more slowly developing pre-adults and lighter adults which are more likely to die during exposure to the 9% ethanol, then the apparently superior development times and adult weights of the 3% lines could also be explained by a bias due to a truncated sampling distribution. Whereas the more slowly developing pre-adults and lighter adults in the 6% and 9% lines may survive the 9% test dose and contribute to the development time and weight estimates, in the 3% lines these flies may die and so not contribute to the estimates.

While the interpretation of the development time and adult weight results of the 3% lines is thus ambiguous, the productivity results for these lines clearly show a degree of tolerance to the 9% test dose. Their productivities were intermediate between that of the 0% and those of the 6% and 9% lines, despite the fact that, as components of productivity, the survival rates of adults and pre-adults in the 3% lines were lower even than those of the 0% lines. This would suggest a compensating advantage in another, unmeasured component of productivity, for example egg-laying behaviour.

Some tolerance to stressful ethanol levels among the 3% lines is also indicated by the fact that they showed the same increased resistance to the gaseous ethanol as did the 6% and 9% lines.

In contrast to all the other components measured, the pre-adult and adult survival rates of the 3% lines clearly showed even less tolerance to the 9% test dose than did those of the 0% lines. Now the longevity analysis of van Herrewege and David (1980), Parsons and Stanley (1981) and Daggard (1981) indicates that low concentrations like 3% ethanol can be a metabolic benefit to *D. melanogaster*. In the present case it would seem that the selection for maximal utilisation of this beneficial ethanol level in the 3% lines involved genetic changes which in some respects were opposite to the changes selected to minimise the detriment due to the stressful levels in the 6% and 9% lines. Confirmation of this interpretation would obviously require further fitness component analyses of the selection lines on a test dose of 3% ethanol in the medium. However, the notion is strongly supported by the data of van Herrewege and David (1980) whose results indicated that two tropical populations sensitive to high ethanol levels were better able to utilise low ethanol levels than was a temperate population tolerant to the high doses.

Thus, our experiment has shown that a complex phenotype is selected on 3% ethanol which in some components is different or opposite to that selected on 6% and 9% ethanol. The work of Starmer et al. (1977) suggests that to some extent tolerance involves coping with interactions between alcohol levels and the yeast and bacterial flora of the media and the gut; so some aspects of the tolerance phenotype may not be directly related to the metabolism of alcohol. Nevertheless, the complexity and concentration dependence of the phenotype have important implications for our understanding of the adaptations by wild *D. melanogaster* to ethanol concentrations ranging from negligible levels to above 12%. Further work is needed not only to compare the genetic bases of tolerance and utilisation, but also to examine the relevance of the two characters to the various ecologies of wild flies.

Finally it remains to relate the present results on ethanol tolerance/utilisation to those in Oakeshott et al. (1984) on *Adh* allele frequency changes in the same selection lines. Here we have reported that fitness on ethanol media responded to selection in a way which was homogeneous among base populations but varied with the ethanol concentration and fitness component tested. Yet a response of *Adh* allele frequency changes over the thirty generations was only obtained in one (Brisbane) of the eight base populations in Oakeshott et al. (1984). And even then it was limited to the 9% selection lines, where the frequency of the *F* allele rose significantly above its frequency in the 0% control lines.

The general failure of *F* frequencies to rise when these freshly collected outbred populations are selected on ethanol media contrasts with the increases in *F* frequency which are regularly observed when inbred and long established laboratory populations are selected in this way (Oakeshott et al. 1984). It also contrasts with the results from single generation tests of tolerance to ethanol media, even among freshly collected outbred populations, in which the tolerance of *Adh FF* and *FS* flies repeatedly exceeds that of *SS* flies (Gibson and Oakeshott 1982; van Delden 1982, for reviews).

A parallel paradox has emerged in recent studies of tolerance to stressful levels of gaseous ethanol. Under these conditions tolerance (i.e. knockdown time as measured in the present experiment) in single generation tests is significantly greater for *SS* than *FF* or *FS* flies (Oakeshott et al. 1980; Cohan and Graf 1985). However, further selection for increased tolerance in freshly collected mass collections does not lead to an increase in *S* frequency (Cohan and Graf 1985). Clearly, in the long term, *Adh* allele frequencies in such populations resist the impetus for change in either direction provided by selection for tolerance to the various ethanol conditions. We suspect that this resistance is due to stabilising selective forces imposed by some function of alcohol dehydrogenase other than the catabolism of environmental ethanol.

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