

Alcohol Dehydrogenase Polymorphism of *Drosophila melanogaster*: Aspects of Alcohol and Temperature Variation in the Larval Environment

Stephen W. McKechnie and Phillip Morgan

Department of Genetics, Monash University, Clayton, Vic. 3168

Abstract

Some natural habitats of *D. melanogaster* larvae were examined for short-chain alcohols by gas–liquid chromatography. All habitats contained a mixture of such alcohols, although ethanol predominated and was found at concentrations ranging from 0.02 to 4.0% (v/v). Normal propanol, propan-2-ol, n-butanol and methanol were nearly always present. Larval-to-adult survival was determined at three temperatures on laboratory media supplemented with alcohol mixtures similar to those found in nature, and using strains which differed in alcohol dehydrogenase genotype. The basic medium was one in which strains compete for limited amounts of dead yeast. Significant genotype–alcohol and genotype–temperature interactions highlighted the sensitivity of this technique for detecting larval fitness variation. The alcohol effect on survival to the adult stage was strongly dependent on temperature—a result which may be of value in our understanding of the ecology of this species.

Extra keywords: genetic polymorphism.

Introduction

Considerable information is available about the alcohol dehydrogenase locus (*Adh*) in *Drosophila melanogaster*—a locus polymorphic in this species throughout the world (see references and discussions in van Delden *et al.* 1978; MacDonald *et al.* 1980 and Oakeshott *et al.* 1981). However, the nature of the balancing selective forces which maintain the polymorphism in natural populations is still not understood. In this study the levels of some naturally occurring environmental alcohols were estimated and the possible influence of these, and of larval development temperature, on survival and genetic variation at the locus were examined.

A major function of alcohol dehydrogenase (ADH) is to metabolize environmental alcohols. A number of laboratory studies have demonstrated the potential of ethanol to select at the locus (see, for example, Gibson 1970; Oakeshott 1976; and Cavener and Clegg 1981). However, it is difficult to extrapolate from these studies to possible selective effects in the field since little is known about the type or levels of alcohols present in natural habitats. ADH catalyses *in vitro* the oxidation of a number of other short-chain primary and secondary alcohols (Dickinson and Sullivan 1975) and these alcohols have also been implicated in fitness determination at the locus (Morgan 1975; Ainsley and Kitto 1975; van Delden *et al.* 1975). If they occur in nature these alcohols may select at the *Adh* locus.

Both field and laboratory studies have implicated environmental temperature as a second factor selecting at the locus (see, for example, Johnson and Powell 1974; McKenzie and McKechnie 1981). Under constant-temperature conditions the life cycle of this species can only be completed within a restricted temperature range (from about 15 to 30°C—see McKenzie 1978). Although short exposures to temperatures outside this range may be tolerated (Parsons 1977; Schenfeld and McKechnie 1979) most natural populations presumably develop at variable temperatures within this range. If ADH plays a significant role in developmental metabolism, and this is indicated by its high levels of activity in larval fat body (Ursprung *et al.* 1970), these permissive temperatures would seem appropriate to use in larval tests for selection at the locus.

Gas-liquid chromatography (GLC) was used to detect short-chain alcohols in some larval habitats of *D. melanogaster* (mainly decomposing fruit), and to give an approximation of the level at which they occurred. Then, in laboratory medium at three temperatures, two different mixtures of alcohols, similar to mixtures found in nature, were tested for possible survival and selective effects during larval development.

Materials and Methods

Estimation of Environmental Alcohols

Seven larval habitats were sampled. Five of these were damaged fruit placed in separate heaps under tree shade in suburban Melbourne at the peak of the *D. melanogaster* season (March). These were sampled for alcohol content after about 8 days of 'maturation' as a larval habitat (when third-instar larvae could be readily detected). Small pieces of rotting fruit were taken from larval sites and either squeezed for juice or centrifuged (without homogenization) to obtain a clear supernatant. These aqueous samples were sealed into a small test tube with Parafilm and deep frozen. Evaporation of alcohols was minimized by keeping tubes in an ice-bath before analysis and unsealing only for short periods. A sixth sample was taken from a pile of grape skin and stalk residues at the Tahbilk Winery, approximately 100 km north of Melbourne. This site, about 200 m south of the Tahbilk cellar, attracts large numbers of *D. melanogaster* and is a rich larval habitat (McKenzie and McKechnie 1979). The samples were from the drier, post-fermentation stage of the decomposing pile. To extract juice from skins a minimum volume of distilled water was added and the pulp squeezed. Finally, red wine seepage was taken from the base of an oak cask in the Tahbilk cellar. For analysis, 5- μ l aliquots were injected into a Hewlett-Packard research chromatograph, model 5750, with dual flame ionization detectors. Routinely, two 1.8 m (3 mm o.d.) stainless-steel columns packed with Porapak Q (120–150 mesh) (Waters Assoc. Inc., Mass., U.S.A.) were used (column temperature 130–240°C, programmed at 6°C/min; carrier gas N₂; flow rate 16 ml/min; injection port and flame detector temperature both 270°C). To distinguish n-butanol from propionic acid a column of 5% (w/w) free fatty acid phase on acid-washed dimethylchlorosilane (Chromosorb W, 80–100) was used. Peak 'spiking' was carried out when necessary. Estimates of alcohol concentration were made by peak height comparison of mixtures of known alcohols made fresh each day and injected at the same attenuations as unknowns. Each sample was injected in duplicate, once before and once after the standard mixture, and the mean peak height compared to its standard. In no duplicate comparison was the taller peak more than 14% higher than the shorter.

The Flies

Two large populations, one homozygous *Adh^S* and the other homozygous *Adh^F*, were established at the same time by pooling, for each, the progeny of 20 pair-matings derived entirely by outbreeding from a population polymorphic at the *Adh* locus. This population was initiated several generations earlier from large numbers of flies caught at Tahbilk (>200♀). The aim was (1) to obtain a large random sample of background genes across the entire genome and (2) to randomize the sample of

Adh^F and *Adh^S* electromorphs of the type discussed by Lewis and Gibson (1978). The large homozygous populations (greater than 200 females per generation) were maintained at 25°C for 11 months prior to the test. Heterozygous individuals were generated from large number of pair-matings between members of these base strains.

Larval-to-adult Viability Test

The basic design of this experiment is similar to that of De Jong and Scharloo (1976). A bottom 5 ml layer of 2% (w/v) agar minimized evaporation from a top feeding layer of 2 ml of 1% (w/v) agar which contained killed yeast (Mauri Brothers & Thomson, Melbourne) at a concentration of 0.06 mg per larvae. In this medium larvae compete for yeast in the presence of alcohols. All vials contained 0.1% (w/v) Nipagin throughout and one of the following three levels of alcohol, supplement throughout both agar layers: (1) control vials with no added alcohols; (2) simulated apple alcohols, where 0.131% of the water was replaced with an alcohol 'cocktail' such that final concentrations (v/v) were 0.005% methanol, 0.02% ethanol, 0.1% n-propanol and 0.006% propan-2-ol; (3) simulated grape pile alcohol vials such that final concentrations (v/v) were 0.04% methanol, 2.50% ethanol, 0.43% n-propanol, 0.06% propan-2-ol and 0.05% n-butanol.

Twenty newly hatched larvae (0–3 h old) of each of the three genotypes were placed into each vial (60 per vial). Vials were transferred to constant-temperature incubators at 18, 25 or 29°C. Three hundred larvae (five vials) for each combination of the three temperatures and three alcohol treatments (including control) were set up, giving a total of 2700 larvae. GLC analysis of the medium in several vials as larval development progressed indicated that alcohol levels in supplemented vials remained higher than in controls, into the third-instar larval stage. Daily records of eclosion number were recorded and genotypes of eclosed adults were determined by electrophoresis on Cellogel.

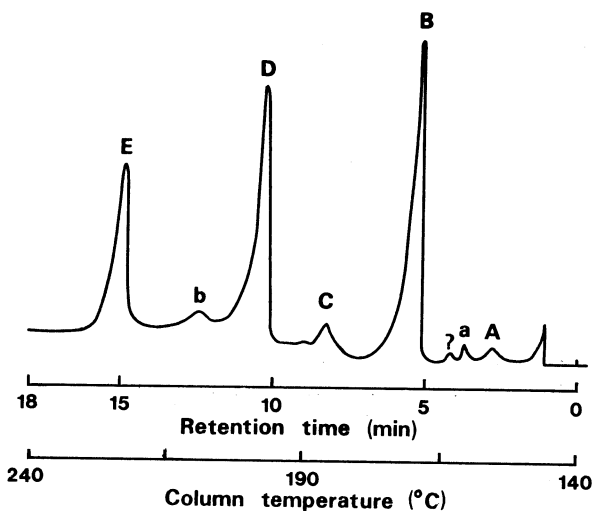


Fig. 1. GLC output of a single 5 μ l pear-rot sample. A, methanol; B, ethanol; C, propan-2-ol; D, n-propanol; E, n-butanol; a, acetaldehyde; b, butylaldehyde.

Results

Estimation of Environmental Alcohols

Five alcohols (methanol, ethanol, n-propanol, n-butanol and propan-2-ol) occurred commonly throughout the seven sample habitats (see Fig. 1 and Table 1). Other alcohols were detected but were both uncommon and present at very low concentration (less than 0.01% v/v). Ethanol was the predominant alcohol, up to a concentration of about 4.0% in rotting grape and 2.5% in grape pile. At the time

of sampling red wine seepage from the bottom ledge of a cask the substrate was fairly dry and mature as a habitat—though larvae were present. Here ethanol was recorded at 0.22%. Two samples which were taken by syringe from inside a seepage source high on a cask gave ethanol levels of 7.0 and 10.5% (larvae absent). Ethanol was detected in every sample although relatively little was present in apple and tomato. The second most common alcohol was n-propanol which occurred in all samples. It was the predominant alcohol in the apple sample (0.1%) and was detected at high levels in grape (1.0%) and banana (0.46%). Propan-2-ol occurred in most samples up to the highest observed level of 0.08% in rotting grape.

Table 1. Range of five alcohol concentrations (% v/v) estimated for seven larval habitats of *D. melanogaster*

Number of samples of each type of habitat used to obtain the range given in parenthesis

Habitat	Methanol	Ethanol	n-Propanol	n-Butanol	Propan-2-ol
Pear (3)	0.03–0.05	0.25–2.20	0.25–0.35	0.06–0.20	0.03–0.07
Banana (4)	0.02–0.06	0.41–0.46	0.13–0.46	0.01–0.17	0.01–0.06
Tomato (3)	0.01–0.04	0.09–0.80	0.02–0.07	<0.005	—
Apple (1)	0.005	0.02	0.10	—	0.006
Grape (4)	0–0.02	1.00–4.0	0.36–1.0	0.03–0.20	0.03–0.08
Grape pile (3)	0.03–0.04	0.50–2.50	0.24–0.43	0.04–0.05	0.04–0.06
Wine seepage ^A (1)	0.007	0.22	0.01	0.005	0.01
Overall range	0–0.06	0.02–4.0	0.01–1.0	0–0.20	0–0.08

^A Dry—see text.

Larval-to-adult Viability Test

For each vial the number of eclosed adults of each genotype was expressed as a proportion of the number of larvae of that genotype set up, i.e. 20. In some vials, especially in the absence of added alcohol, few or no adults eclosed. Since 20 larvae were set up in these vials (and it was known that other treatments in the same experiment facilitated survival), the data were analysed by the factorial analyses of variance (ANOVA), which were carried out on the angular transformation of these proportions. The results are summarized in Figure 2 and Table 2.

Genetics effects

Survival was not independent of genotype or, more correctly, strain. The ANOVA (Table 2) indicated a highly significant main effect of genotype. Notwithstanding a significant genotype–temperature interaction, fewer FF flies eclosed under all conditions tested (Fig. 2), and heterozygotes performed best under most conditions.

A significant genotype–temperature interaction, which in this ANOVA was the only significant interaction involving genotype (there was no three-way interaction), appeared to be largely due to the relatively higher survival of heterozygotes at 25°C, compared with those surviving at the higher and lower test temperatures. This is also indicated in Fig. 2 and was supported by a second ANOVA (Table 2) in which heterozygotes were omitted from the analysis. In this case the interaction disappeared. A significant genotype–alcohol interaction occurred in the second ANOVA and indicated that the relative survival of the homozygous strains changed with the alcohol content of the medium. Because of low survival in control vials and the obvious

overall differences in survival among strains (differences which seem unlikely to be attributable to the *Adh* locus) further interpretation of specific genotype interactions which might have occurred has not been attempted.

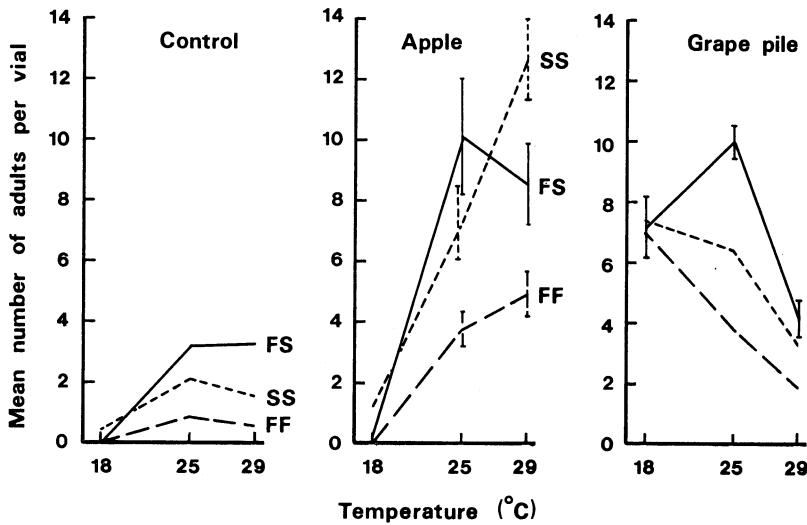


Fig. 2. Survival to adult stage of the three *Adh* genotypes (*Adh^F*, *Adh^S*, *Adh^{FS}*) under various conditions of temperature and alcohol supplement; some representative standard errors (\pm) are shown.

Table 2. Factorial analyses of variance of the effects of genotype, alcohol and temperature on the proportion surviving per replicate (after angular transformation)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Source of variation	(a) All three <i>Adh</i> genotypes			(b) Two <i>Adh</i> homozygotes only		
	D.F.	M.S.	<i>F</i> ratio	D.F.	M.S.	<i>F</i> ratio
Main effects						
Genotype	2	1303.46	21.99***	1	1696.85	28.57***
Alcohol	2	4998.22	84.32***	2	3358.86	56.55***
Temperature	2	2760.03	46.56***	2	972.19	16.37***
Two-way interactions						
Genotype–alcohol	4	139.87	2.36	2	246.75	4.15*
Genotype–temperature	4	288.02	4.86**	2	86.79	1.46
Alcohol–temperature	4	2223.92	37.52***	4	1540.45	25.93***
Three-way interaction						
Genotype–temperature–alcohol	8	49.87	0.84	4	36.73	0.62
Error	108	59.27		72	59.40	

Ecological effects

The various combinations of temperature and alcohol have had different effects on overall survival to the adult stage. This is indicated by the highly significant alcohol–temperature interactions (Table 2). Because of interactions involving genotype, specific interpretations are difficult; however, one obvious effect was a

marked increase in survival at 18°C, when grape-pile alcohols were present (see Fig. 2). This effect was not produced at 18°C by the lower level of alcohols in apple supplement. These low levels, however, gave markedly enhanced survival at the higher temperatures. This increase in survival at 29°C with the apple alcohol mixture appeared not to occur with higher alcohol levels of grape-pile supplement. On grape-pile supplement a temperature increase to 29°C brought about a decrease in survival.

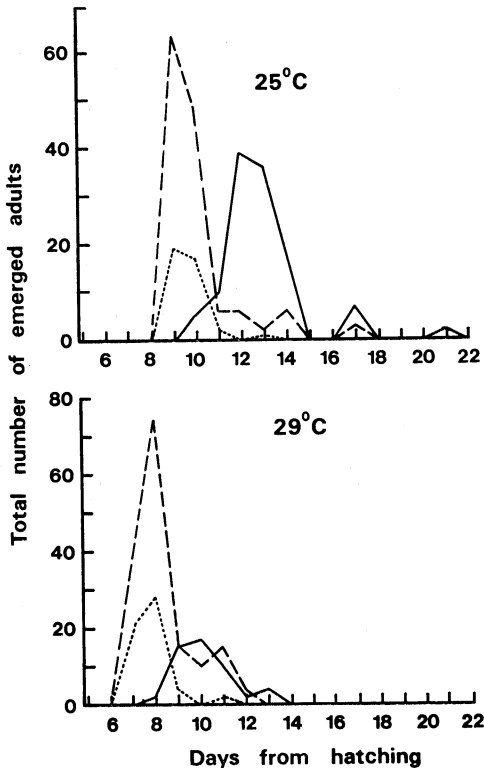


Fig. 3. Effect of temperature and alcohol supplement on egg-to-adult development rate. Upper, 25°C. Lower, 29°C. Control. ----- Apple. ——— Grape pile.

Another conspicuous effect of the grape-pile alcohol was a delay in development time (Fig. 3). This occurred at the two higher temperatures, 25° and 29°C, and was indicated at 18°C where adult yields were low—especially on control medium.

Discussion

Among the small number of habitats sampled the data indicate considerable variation in both the amount and type of various short-chain alcohols in the environment of *D. melanogaster* larvae. In all samples ethanol was found as one of the components of a mixture of alcohols. It was, however, almost always the predominant alcohol (in terms of % v/v). Higher levels of ethanol have been previously reported for a *D. melanogaster* larval habitat—from the 'fermentation stage' of the same grape pile sampled in this study (McKenzie and McKechnie 1979). The present authors suspect, however, that the levels of from 0.02 to 4.0% reported in this paper are more typical for those south-eastern Australian populations which are commonly found around fruit distribution centres and in orchards on wind-fallen fruit and

discarded heaps. Though propan-2-ol was very often present, it was not found at levels as high as those of the primary alcohols (in terms of % v/v).

It was decided to look for effects of alcohol from a typical larval habitat such as the grape-pile (total alcohol concentration 3.08%) and from a second habitat with a lower total alcohol concentration (0.13%). In the latter case a simulated apple-alcohol mixture, where n-propanol predominated, was used. The constant temperatures chosen encompassed the range in which normal larval and pupal development proceeds without incurring mortality (McKenzie 1978). Except for these conditions of alcohol and temperature, the medium used (limited amounts of dead yeast) is perhaps unlike that of any natural habitat. However, this medium was chosen to accentuate small differences in development rate which may occur among *Adh* genotypes (van Delden and Kamping 1979). In a situation where larvae of this species compete for limited amounts of yeast individuals which grow faster are at an advantage. When the food resource is depleted it is only those larvae which reach the critical minimum weight essential for pupation that survive to the adult stage. Slower developing larvae fail to reach this weight and perish as small larvae (Bakker 1961). Small differences in developmental rate may constitute an important component of fitness variation in a continuously breeding species such as *D. melanogaster* (Lewontin 1965, 1974). Thus any survival differences detected using this methodology may be of consequence for the maintenance of genetic variation in natural populations. The approach has been previously used in this species to demonstrate fitness variation among enzyme genotypes (De Jong and Scharloo 1976; McKechnie *et al.* 1981).

Genotype fitness variation in this study is not convincingly relatable directly to the *Adh* locus. Such large overall differences in total survival among the strains is hardly attributable to changes of a single *Adh* allozyme. The differences might be attributable to differential changes in frequencies of background genes which occurred in the two homozygous base strains during the period prior to testing. Such differences could produce associated overdominance and be largely responsible for the overall higher survival of heterozygotes. Also, none of the reported temperature-dependent biochemical properties of ADH allozymes, especially those carried out using *Tahbilk* flies (McKenzie and McKechnie 1981), would lead one to predict the observed relative increase in heterozygote performance at 25°C (Vigue and Johnson 1973; Day *et al.* 1974).

Nonetheless, it remains a possibility that ADH may have contributed to changes in relative survival of the strains. Genotype-temperature and genotype-alcohol interactions are previously implicated properties of this locus (Oakeshott 1976; van Delden *et al.* 1978; Malpica and Vassallo, 1980; McKechnie *et al.* 1981; McKenzie and McKechnie 1981). Also, previously described biochemical properties of the allozymes, which have failed to be predictive of survival in this test, have been carried out using single alcohol substrates. Such parameters may not be relevant to metabolism of mixtures of environmental alcohols as used in this test (Ainsley and Kitto 1975).

The significant temperature- and alcohol-related variation in total adult survival levels and the effect of grape-pile alcohols on developmental rate (pooling across genotype) indicates the possible importance of these factors as determinants of survival in nature, especially if larval competition for food occurs. Certainly, the value of ethanol as an adult and larval resource has been previously demonstrated

under laboratory conditions (Parsons *et al.* 1979). Results in the present study suggest that a total alcohol concentration of about 3% (of which 2.5% was ethanol in the grape-pile supplement) may be a particularly valuable resource at cooler temperatures (18°C in this study). A lower total alcohol concentration ($\approx 0.1\%$) may be valuable at higher temperatures (29°C). These findings only hint at possible effects in nature because they occur in the laboratory under specific conditions of competition for dead yeast. The retardation of developmental rate observed here on grape-pile alcohols was not observed by Parsons *et al.* (1979) on a standard laboratory medium supplemented with 3% ethanol. Thus effects of alcohol on developmental rate and survival in nature may depend on the particular mixture of alcohols and/or on levels of available nutrition.

Data of this study confirm that mixtures of ethanol with other short-chain alcohols are a common component of the larval habitat of this species and that these alcohols, at the concentrations at which they occur, potentially constitute important determinants of survival. Any such effects are likely to depend on temperature and perhaps on nutritional availability. The approach of competing genotypes for limited amounts of dead yeast appears to be a sensitive method of detecting fitness variation among larval *D. melanogaster* genotypes. It is suggested that it could be valuably employed in future studies, using larvae from polymorphic outbred populations, to assess the effects of temperature, of low levels of ethanol, and of alcohol mixtures, on *Adh* fitness variation.

Acknowledgments

The authors thank Professor Peter Parsons and Drs John McKenzie and Lincoln Schmitt for valuable discussions and both Dr John Oakeshott and a referee for constructive comments on the manuscript. We thank Leanne Ritchie and Elizabeth Mason for competent technical assistance and the Monash Chemistry Department for use of their GLC facilities. The Australian Research Grants Committee provided financial support.

References

- Ainsley, R., and Kitto, B. (1975). Selection mechanisms maintaining alcohol dehydrogenase polymorphisms in *Drosophila melanogaster*. In 'Isozymes'. (Ed. C. L. Markert.) Vol. 2. pp. 733-43. (Academic Press: New York.)
- Bakker, K. (1961). An analysis of factors which determine success in competition for food among larvae of *Drosophila melanogaster*. *Arch. Neerl. Zool.* **14**, 200-81.
- Cavener, D. R., and Clegg, M. T. (1981). Multigenic response to ethanol in *Drosophila melanogaster*. *Evolution* **35**, 1-10.
- Day, T. H., Hillier, P. C., and Clarke, B. C. (1974). Properties of genetically polymorphic isozymes of alcohol dehydrogenase in *Drosophila melanogaster*. *Biochem. Genet.* **11**, 141-53.
- De Jong, G., and Scharloo, W. (1976). Environmental determination of selective significance or neutrality of amylase variants in *Drosophila melanogaster*. *Genetics* **84**, 77-94.
- Delden, W. van, Kamping, A., and van Dijk, H. (1975). Selection at the alcohol dehydrogenase locus in *Drosophila melanogaster*. *Experientia* **31**, 418-19.
- Delden, W. van, Boerema, A. C., and Kamping, A. (1978). The alcohol dehydrogenase polymorphism in populations of *Drosophila melanogaster*. I. Selection in different environments. *Genetics* **90**, 161-91.
- Delden, W. van, and Kamping, A. (1979). The alcohol dehydrogenase polymorphism in populations of *Drosophila melanogaster*. 3. Differences in developmental times. *Genet. Res. Camb.* **33**, 15-27.

- Dickinson, W. J., and Sullivan, D. T. (1975). 'Gene-Enzyme Systems in *Drosophila*.' (Springer-Verlag: New York.)
- Gibson, J. (1970). Enzyme flexibility in *Drosophila melanogaster*. *Nature (London)* **227**, 959-61.
- Johnson, F. M., and Powell, A. (1974). The alcohol dehydrogenase of *Drosophila melanogaster*: Frequency changes associated with heat and cold shock. *Proc. Natl Acad. Sci. USA* **71**, 1783-4.
- Lewis, N., and Gibson, J. (1978). Variation in amount of enzyme protein in natural populations. *Biochem. Genet.* **16**, 159-70.
- Lewontin, R. C. (1965). Selection for colonising ability. In 'The Genetics of Colonising Species'. (Eds H. G. Baker and G. L. Stebbins.) pp. 77-91. (Academic Press: New York.)
- Lewontin, R. C. (1974). 'The Genetic Basis of Evolutionary Change.' p. 62. (Columbia University Press: New York.)
- Malpica, J. M., and Vassallo, J. N. (1980). A test for the selective origin of environmentally correlated allozyme patterns. *Nature (London)* **286**, 407-8.
- McDonald, J. F., Anderson, S. M., and Santos, M. (1980). Biochemical differences between products of the *Adh* locus in *Drosophila*. *Genetics* **95**, 1013-22.
- McKechnie, S. W., Kohane, M., and Phillips, S. C. (1981). A search for interacting polymorphic enzyme loci in *Drosophila melanogaster*. In 'Genetic Studies of *Drosophila* Populations'. (Eds J. B. Gibson, and J. G. Oakeshott.) pp. 121-38. (Australian National University: Canberra.)
- McKenzie, J. A. (1978). The effect of developmental temperature on population flexibility in *Drosophila melanogaster* and *D. simulans*. *Aust. J. Zool.* **26**, 105-12.
- McKenzie, J. A., and McKechnie, S. W. (1979). A comparative study of resource utilisation in natural populations of *Drosophila melanogaster* and *D. simulans*. *Oecologia (Berl.)* **40**, 299-309.
- McKenzie, J. A., and McKechnie, S. W. (1981). The alcohol dehydrogenase polymorphism in a vineyard cellar population of *Drosophila melanogaster*. In 'Genetic Studies of *Drosophila* Populations'. (Eds J. B. Gibson, and J. G. Oakeshott.) pp. 201-15. (The Australian National University: Canberra.)
- Morgan, P. D. (1975). Selection acting directly on an enzyme polymorphism. *Heredity* **35**, 124-7.
- Oakeshott, J. G. (1976). Selection at the alcohol dehydrogenase locus in *Drosophila melanogaster* imposed by environmental alcohol. *Genet. Res.* **26**, 265-74.
- Oakeshott, J. G., Gibson, J. B., Anderson, P. R., Knibb, W. R., Anderson, D. G., and Chambers, G. K. (1981). Alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase clines in *Drosophila melanogaster* on three continents. *Evolution* **35**, in press.
- Parsons, P. A. (1977). Resistance to cold temperature stress in populations of *Drosophila melanogaster* and *D. simulans*. *Aust. J. Zool.* **25**, 693-8.
- Parsons, P. A., Stanley, S. M., and Spence, G. E. (1979). Environmental ethanol at low concentrations: longevity and development in the sibling species *Drosophila melanogaster* and *D. simulans*. *Aust. J. Zool.* **27**, 747-54.
- Schenfeld, E., and McKechnie, S. W. (1979). The α -glycerophosphate dehydrogenase polymorphism in *Drosophila melanogaster*: adult survival under temperature stress. *Theoret. Appl. Genet.* **54**, 235-7.
- Ursprung, H., Sofer, W. H., and Burroughs, N. (1970). Ontogeny and tissue distribution of alcohol dehydrogenase in *Drosophila melanogaster*. *Wilhelm Roux' Arch.* **164**, 201-8.
- Vigue, C. L., and Johnson, F. M. (1973). Isozyme variability in species of the genus *Drosophila*. VI. Frequency-property-environment relationships of allelic alcohol dehydrogenase in *D. melanogaster*. *Biochem. Genet.* **9**, 213-27.

