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Esterase-6 polymorphism in *Drosophila melanogaster*: Effects of temperature and methyl malonate on genotypic trajectories in polymorphic populations set up with highly inbred lines

RODOLFO COSTA[†], LAURA ZONTA[+], SURESH D JAYAKAR [+] and LOREDANA NIGRO[†]

[†] Department of Biology, University of Padua, Via Loredan 10, 35100 Padova, Italy

[+] The "A. Buzzati-Traverso" Department of Genetics and Microbiology, University of Pavia, Via S. Epifanio 14, 27100 Pavia, Italy

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Abstract. It is generally difficult to identify possible effects of selection at a specific locus because of the heterogeneity of the genetic background. Geographical patterns of *Est-6* gene frequencies suggest that there is selection at this locus but selection on loci closely linked to it cannot be excluded. Differences in catalytic properties between allozymes have been shown *in vitro*; further, several laboratory studies have shown apparent fitness differences between allozymes. Our study used inbred lines highly homogeneous in the genetic background. Four populations were set up from *Est-6*^{S} and *Est-6*^{F} homozygous females inseminated by males of the same genotype at each combination of three factors: temperature (18 and 25°C); methyl malonate (presence or absence); input gene frequencies [$p(S) = 0{\cdot}2$ and $0{\cdot}8$]. The populations were sampled periodically for about 28 generations. Methyl malonate was chosen to exert pressure in the enzymatic function of esterase-6. Statistical analyses show that: there are no sex differences; gene frequencies change from input values to those of the first sampling, when only individuals of the first generation are present at 18°C or individuals of the second generation just begin to appear at 25°C; gene frequencies do not change thereafter and Hardy-Weinberg equilibrium is established. The changes in gene frequencies observed in the first generations suggest that *Est-6* can under certain conditions be a target of selection. Such conditions may not, however, occur in natural populations.

Keywords. Esterase-6 polymorphism; highly inbred lines; methyl malonate; selection.

1. Introduction

Different allozymes at a particular locus may be selectively neutral and simply reflect the consequences of random drift within populations, migration or hitch-hiking. The adaptive significance of a polymorphism at a given locus must be supported by consistent experimental results which indicate causal relationships between environmental factors and genetic variability at that locus. Moreover such results at the population level have to be complemented by studies of the biochemical kinetics and the physiological function of the different allozymes as suggested by Nevo *et al* (1984).

The most intriguing problem that arises in the experimental approach to the analysis of a polymorphism is due to the almost complete impossibility of excluding an influence of the genetic background. Linkage and a consequent gametic disequilibrium could account for many results apparently suggesting selection at a particular polymorphic locus though this phenomenon was not seen to be

widespread in natural populations (for references concerning studies in Drosophila, see Costa *et al* 1985) as might have been expected from the theoretical results of Franklin and Lewontin (1970).

Various authors have used different methods in order to minimize the effects of heterogeneity in the genetic background, mainly by using either genetically randomized lines or inbred lines (Wills and Nichols 1971; Wills *et al* 1975; De Jong and Scharloo 1976; Bijlsma 1978; Hoorn and Scharloo 1981; Palabost-Charles 1982; Yamazaki *et al* 1983; Costa *et al* 1985; Nigro *et al* 1985) and the application of a specific pressure on the enzymatic function (Gibson 1970; De Jong and Scharloo 1976; Cavener and Clegg 1978; Danford and Beardmore 1980; Bijlsma and Kerver 1983; Powell and Andjelkovic 1983; Costa *et al* 1985).

In *Drosophila melanogaster*, among other polymorphisms for gene-enzyme systems which have been suggested as being adaptive (ADH: van Delden *et al* 1975, 1978, Cavener and Clegg 1978, 1981a; α-GPDH: Schenfeld and McKechnie 1979, McKechnie *et al* 1981; G-6-PDH and 6-PGDH: Bijlsma 1978, Cavener and Clegg 1981b; Amy: Powell and Andjelkovic 1983) the one at the *Est-6* locus has been the object of considerable interest, particularly during the past few years. This work was stimulated by the fact that information on gene frequency data in the wild, molecular properties, the metabolic role and laboratory population tests have become available.

The *Est-6* locus has been mapped to 3–36·8 on the genetic map (Wright 1963) and cytologically at 69 A 1–5 (Akam *et al* 1978). X-linked regulatory loci, polymorphic in natural populations, have been detected (Tepper *et al* 1982, 1984) and a locus (3–56·7) has been found which causes post-translational modifications of some esterase-6 allozymes (Cochrane and Richmond 1979). Two common electrophoretic alleles (*Est-6*^{S} and *Est-6*^{F}) have been found in natural populations. Moreover, rare electrophoretic alleles and isoelectrophoretic alleles which alter heat stability, substrate specificity and activity have been described (see references in Costa *et al* 1982).

Anderson and Oakeshott (1984) have described parallel patterns of geographical variation for the homologous esterase-6 polymorphisms in the sibling species *D. melanogaster* and *D. simulans*. The latitudinal cline observed over a vast geographical area covering Australasia, North America and Europe with a reversed trend in the two hemispheres for both species would be difficult to attribute to random processes. The geographical patterns described could be due to selection acting on *Est-6* itself or on closely linked loci. Anderson and Oakeshott (1984) argued that if selective processes act on linked genes, then disequilibrium should have been established before separation and maintained by epistatic selection in both species after divergence. It is suggested that unless the *Est-6* locus is part of a block of genes involved in such epistatic selection, the observed geographical patterns are due to selection involving the *Est-6* locus.

Biochemical characterizations of partially purified esterase-6 (EST-6) allozymes have been performed by Danford and Beardmore (1979) and Costa *et al* (1983). Differences in specific activity between allozymes for a number of synthetic esters and at different temperatures have been observed but, since the *Kcat* values are not available, the results could be at least partially due to differences in enzyme concentration. Information on physiochemical and kinetic properties of purified EST-6^S and EST-6^F allozymes have also been published (Mane *et al* 1983a). Esterase-6

is a monomeric glycoprotein (62,000–65,000 D) with a single active catalytic site. No differences in catalytic efficiency of the two allozymes have been found at 30°C but in one case there were significant differences in *Kcat-Km* ratios for the substrate β-naphthol propionate which suggests that the two allozymes may differ in substrate specificity.

EST-6 together with an *in vivo* substrate (*cis*-vaccenyl acetate) is a component of the seminal fluid and is involved in reproduction (Mane *et al* 1983b). Moreover EST-6 is potentially involved in the metabolism of esters derived from external substrates (Danford and Beardmore 1979, 1980; Zera *et al* 1983).

Several studies carried out in the laboratory suggest that there are differences in fitness components between the allozymes at the *Est-6* locus (Wright 1963; MacIntyre and Wright 1966; Kojima and Yarbrough 1967; Birley and Beardmore 1977; Costa and Beardmore 1980; Gilbert and Richmond 1982; Nigro *et al* 1985), but generally in these studies it is not possible to exclude the influence of the genetic background, especially when the populations used in the experiments were established from only a few lines as pointed out by Jones and Yamazaki (1974). Moreover, opposite results have been obtained by Dolan and Robertson (1975) and by Yamazaki *et al* (1983).

Danford and Beardmore (1980) analyzed the effects on larva to adult viability of the addition in the culture medium of three esters known to be rapidly hydrolyzed *in vitro* by esterase-6: diethyl oxalate, methyl malonate and *n*-propyl formate. They found that diethyl oxalate had no effect, methyl malonate was apparently generally toxic, while *n*-propyl formate affected the viability of the genotypes differentially. They used outbred *Est-6*^{S} and *Est-6*^{F} homozygous lines in their experiments and could not therefore exclude the effects of linked loci.

Costa *et al* (1985) analyzed the effects of two of the chemicals studied by Danford and Beardmore, namely *n*-propyl formate and methyl malonate, on the egg to adult viability in polymorphic populations set up using highly inbred *Est-6* lines. These lines were obtained by an inbreeding procedure lasting 100 generations starting with Danford and Beardmore's outbred lines. A detailed scheme of the procedure is described in Costa *et al* (1985). The same paper also presents the results of a computer simulation of this scheme which showed that the genetic background after 100 generations must have reached a very high level of homogeneity. This was confirmed by electrophoretic analysis of allozymes coded by loci linked and unlinked to *Est-6*. The results confirmed some of Danford and Beardmore's (1980) findings, for instance the general reduction of yield as a consequence of the addition of methyl malonate and the effects on the genotypic output produced by *n*-propyl formate. Moreover methyl malonate also showed differences in the genotypic proportions at the two concentrations adopted. In general the input genotypic composition and the presence of the esters were seen to significantly affect the output genotypic ratios with a different pattern in relation to the nature of the ester and to the presence or absence of heterozygotes in the populations.

Here we present results concerning the effects on the dynamics of *Est-6* polymorphism for two kinds of culture media (a control medium and a medium with 0·05 M methyl malonate added), two temperatures (18°C and 25°C) and two *Est-6*^{S} input frequencies (0·8 and 0·2). Thirty two populations, each with 1000 inseminated females, were started as described in the next section. Populations were started with the same highly inbred lines used by Costa *et al* (1985). Methyl

malonate was used as substrate in order to exert a pressure on the enzymatic function since one of the cleavage products of the substrate (malonate) can inhibit succinate dehydrogenase and cause negative effects on respiratory efficiency (Costa *et al* 1985).

2. Material and methods

2.1 *Inbred lines used*

The inbred lines used in the experiments described here have been obtained adopting a procedure of inbreeding lasting 100 generations as described in the Introduction (Costa *et al* 1985).

2.2 *Design of experiments*

A total of 32 laboratory populations were set up each with 1000 three–four day old females inseminated by males of the same age and genotype. The populations were established at the same time with flies from *Est-6*^{S} and *Est-6*^{F} homozygous inbred lines. Two genetic compositions were used, $p(S) = 0{\cdot}8$ and $0{\cdot}2$. The population cages used had a capacity of more than 4000 adults. Thirty two populations were set up, i.e., 4 replicates of each of 8 experimental conditions, namely all the combinations of three factors: temperature (18 and 25°C), treatment (presence or absence of methyl malonate in the medium), and input gene frequencies [$p(S = 0{\cdot}8$ and $0{\cdot}2$].

The "control" populations (C) were cultured on a sucrose-yeast medium (Mittler and Bennet 1962) while for the "treated" populations (MM) methyl malonate (Sigma Chem. Co.) was added to the medium at a concentration of 0·05 M after cooling the mixture to 40°C. Ten bottles, each containing 80 ml of medium, were inserted at the bottom of each population cage. Five of these were replaced by bottles with fresh medium every 20 days (at 25°C) or 28 days (at 18°C). The populations were maintained for about 400 days at 25°C and 600 days at 18°C.

Cycles of 16 hr light, 8 hr darkness were employed. During the experiments five samples were taken from each population at 25°C, and six samples from those at 18°C. Each sample consisted of 150–200 individuals. The specimens were sexed, the sexes kept separately at 24°C for 3 days and then stored at –30°C until their genotypes were ascertained by electrophoresis. This was done in order to avoid possible effects on the electrophoretic patterns of the presence of the male EST-6 possibly present in the female reproductive tract. Moreover, as both sexes reach a peak of esterase-6 activity 3 to 4 days after emergence, this procedure ensured that all the individuals (including those recently emerged) gave clear results.

Electrophoresis and staining procedures were as previously reported (Costa and Beardmore 1980).

2.3 *Statistical analysis*

The *G*-test was applied when genotypic or allele frequencies were compared. A two-way analysis of variance with replicates was used to analyze the effects of

treatment and temperature either on gene frequencies within a single sample or on gene frequency differences between two samples. The application of the angular transformation to the gene frequencies did not modify the conclusions in any way.

3. Results

Tables 1–4 show the data pooled for the sexes. In fact, of the 175 samples only 14 showed a difference between the genotypic frequencies of males and females ($P = 0{\cdot}057$ of obtaining by chance 14 or more significant G values out of 175).

Since the experiment was started with homozygous females inseminated by males of the same genotype, the first generation could only produce homozygotes. In the first sample at 18°C, obtained when only individuals of the first generation are present, there are only homozygotes, while at 25°C individuals of generation two begin to appear and a few heterozygotes are present.

By the second sample (after about 4 generations), with the exception of the treated (MM) replicates at 25°C, almost all the other populations (126 out of 135) showed Hardy-Weinberg (H-W) equilibrium ($P = 0{\cdot}14$). The treated populations at 25°C took longer to reach H-W equilibrium (third sample). Figure 1 shows the genotypic trajectories for all the replicates.

Figures 2 and 3 on the other hand show the dynamics of the gene frequency of the *Est-6*^{S} allele.

In the analysis we have considered three stages of the experiment: (a) the first sample; (b) the changes occurring between the first and second samples; (c) those occurring from the second sample onwards.

3.1 *The first sample*

During this phase of the experiment, although a few heterozygotes were present at 25°C, we can consider the genotypic frequencies of the two homozygotes, *SS* and *FF*. One thing that is fairly clear (figure 2 and 3; tables 1, 2, 3) is that at the input frequency of 0·8 at 18°C, the frequency of *SS* increases in all four control replicates, while it decreases in all four treated replicates. Again at the input frequency of 0·8, among the control populations, the frequency of the *SS* homozygotes increases in all four, while 3 of the 4 populations at 25°C show a decrease in this frequency (table 5). However, while the former result is highly significant (Fisher's exact test $P = 0{\cdot}014$),the latter is not ($P = 0{\cdot}071$).

The two-way (treatment and temperature) analysis of variance of gene frequencies gives different results for the two input frequencies: for $p(S) = 0{\cdot}8$ there is a highly significant interaction between the effects of the two factors ($F = 11{\cdot}66$; $P = 0{\cdot}01$), while for $p(S) = 0{\cdot}2$ even the single factor F are not significant.

3.2 *Changes occurring between the first and second samples*

In order to find whether any change in gene frequency had taken place between the first sample and the second, a two-way analysis of variance was carried out on the

Table 1. Genotypic compositions, as from electrophoretic analysis, in control populations (C) reared at 18°C. Data over sexes are pooled. Time of sampling is given in days from the setting up of the populations.

Time of sampling	Input $p(S)$	Control populations (18°C)	Genotypes *SS*	*SF*	*FF*	*Est-6*^{S} gene frequency
40	0·8	1	162	—	16	0·910
		2	165	—	13	0·927
		3	163	—	14	0·921
		4	126	—	28	0·818
	0·2	5	72	—	120	0·375
		6	68	—	114	0·374
		7	31	—	129	0.194
		8	32	—	143	0·183
100	0·8	1	107	21	0	0·918
		2	97	25	1	0·890
		3	99	41	0	0·854
		4	113	40	2	0·858
	0·2	5	11	75	65	0·321
		6	15	77	63	0·345
		7	4	47	104	0.177
		8	3	46	98	0·177
200	0·8	1	131	25	0	0·920
		2	107	34	1	0·873
		3	91	25	5	0·855
		4	91	43	4	0·815
	0·2	5	24	59	73	0·343
		6	22	76	49	0·408
		7	4	48	93	0.193
		8	8	51	97	0·215
296	0·8	1	125	30	1	0·897
		2	132	24	0	0·923
		3	115	37	3	0·861
		4	95	54	7	0·782
	0·2	5	23	76	57	0·391
		6	30	71	55	0·419
		7	4	39	86	0.182
		8	11	52	93	0·237
429	0·8	1	125	26	2	0·901
		2	122	22	0	0·923
		3	110	46	3	0·836
		4	100	53	3	0·810
	0·2	5	15	84	55	0·370
		6	18	67	53	0·373
		7	4	41	99	0.170
		8	3	41	100	0·163
571	0·8	1	122	30	3	0·883
		2	146	10	0	0·967
		3	98	54	3	0·806
		4	79	68	9	0·724
	0·2	5	21	63	66	0·350
		6	38	82	36	0·506
		7	2	36	67	0.190
		8	4	49	48	0·282

Table 2. Genotypic compositions in treated populations (MM) reared at 18°C. See also legend to table 1.

Time of sampling	Input $p(S)$	Treated populations (18°C)	Genotypes *SS*	*SF*	*FF*	$Est\text{-}6^S$ gene frequency
48	0·8	1	112	—	44	0·718
		2	112	—	44	0·718
		3	122	—	34	0·782
		4	113	—	43	0·724
	0·2	5	25	—	131	0·160
		6	16	—	137	0·105
		7	28	—	127	0.181
		8	41	—	115	0·263
81	0·8	1	77	81	23	0·649
		2	78	61	13	0·714
		3	83	65	6	0·750
		4	80	62	14	0·712
	0·2	5	2	31	123	0·112
		6	6	31	117	0·140
		7	2	47	104	0·167
		8	8	52	93	0·222
177	0·8	1	72	48	7	0·756
		2	69	47	7	0·752
		3	103	41	9	0·807
		4	49	54	20	0·618
	0·2	5	0	26	90	0·112
		6	5	53	90	0·213
		7	3	23	95	0.120
		8	9	29	54	0·255
273	0·8	1	79	65	9	0·728
		2	82	60	13	0·722
		3	107	41	8	0·817
		4	48	75	31	0·555
	0·2	5	4	24	126	0·103
		6	3	34	119	0·128
		7	3	39	114	0.144
		8	6	43	96	0·189
406	0·8	1	94	55	7	0·778
		2	97	62	5	0·780
		3	99	52	7	0·791
		4	49	72	32	0·555
	0·2	5	3	33	113	0·130
		6	3	46	102	0·172
		7	2	28	111	0.113
		8	8	47	88	0·220
548	0·8	1	117	48	9	0·810
		2	80	69	6	0·738
		3	63	47	5	0·752
		4	18	63	40	0·409
	0·2	5	0	35	59	0·186
		6	1	29	113	0·108
		7	—	—	—	—
		8	2	18	81	0·108

Table 3. Genotypic compositions in control populations (C) reared at 25°C. See also legend to table 1.

Time of sampling	Input $p(S)$	Control populations (25°C)	Genotypes *SS*	*SF*	*FF*	*Est-6^S* gene frequency
26	0·8	1	110	1	44	0·713
		2	121	1	34	0·779
		3	121	5	30	0·792
		4	129	0	26	0·832
	0·2	5	33	0	123	0·211
		6	27	1	127	0·177
		7	25	1	129	0.164
		8	37	0	115	0·243
57	0·8	1	87	57	11	0·745
		2	91	52	12	0·755
		3	115	36	2	0·869
		4	103	47	6	0·811
	0·2	5	7	53	95	0·216
		6	4	40	112	0·256
		7	7	41	107	0.177
		8	1	57	97	0·190
152	0·8	1	69	60	18	0·673
		2	64	74	16	0·655
		3	83	59	13	0·725
		4	93	56	5	0·785
	0·2	5	4	37	113	0·146
		6	3	48	105	0·173
		7	5	46	103	0.181
		8	9	46	96	0·211
236	0·8	1	80	59	12	0·725
		2	65	76	14	0·664
		3	74	67	15	0·689
		4	46	53	11	0·659
	0·2	5	6	50	96	0·203
		6	4	38	113	0·148
		7	7	42	101	0.186
		8	6	46	95	0·197
371	0·8	1	66	73	16	0·661
		2	40	88	28	0·538
		3	53	81	21	0·603
		4	105	48	3	0·826
	0·2	5	10	54	92	0·237
		6	2	30	120	0·111
		7	5	41	107	0.166
		8	6	47	102	0·190

difference between the gene frequencies. Separate analyses were made for the two input frequencies. No significant difference was found either between treatments or between temperatures at either input frequency (table 6).

Table 4. Genotypic compositions in treated populations (MM) reared at 25°C. See also legend to table 1.

Time of sampling	Input $p(S)$	Treated populations (25°C)	Genotypes *SS*	*SF*	*FF*	*Est-6*^{S} gene frequency
22	0·8	1	111	2	42	0·722
		2	123	5	25	0·820
		3	115	0	39	0·747
		4	120	1	35	0·772
	0·2	5	27	2	125	0·182
		6	24	2	123	0·168
		7	32	2	122	0.211
		8	27	2	119	0·189
53	0·8	1	104	30	22	0·763
		2	109	29	18	0·792
		3	94	37	24	0·726
		4	89	34	33	0·679
	0·2	5	16	18	119	0·163
		6	13	26	116	0·168
		7	13	25	121	0.160
		8	12	16	128	0·102
148	0·8	1	66	73	13	0·674
		2	69	61	11	0·705
		3	71	60	22	0·660
		4	82	46	11	0·755
	0·2	5	3	31	120	0·120
		6	2	30	123	0·109
		7	8	49	99	0.208
		8	4	42	108	0·162
232	0·8	1	73	57	10	0·725
		2	67	68	20	0·651
		3	67	70	19	0·653
		4	83	66	6	0·748
	0·2	5	8	35	110	0·166
		6	1	19	65	0·123
		7	6	47	101	0.191
		8	2	25	111	0·105
367	0·8	1	80	57	9	0·743
		2	77	63	16	0·695
		3	59	82	15	0·641
		4	89	57	8	0·762
	0·2	5	5	55	96	0·208
		6	0	27	108	0·100
		7	0	45	100	0 155
		8	3	36	116	0·135

3.3 *Changes occurring from the second sample onwards*

The same analysis was carried out to assess the difference between the gene frequencies in the second sample and those at the fifth and sixth samples at 25°C and 18°C respectively. Again no significant difference was found (table 7).

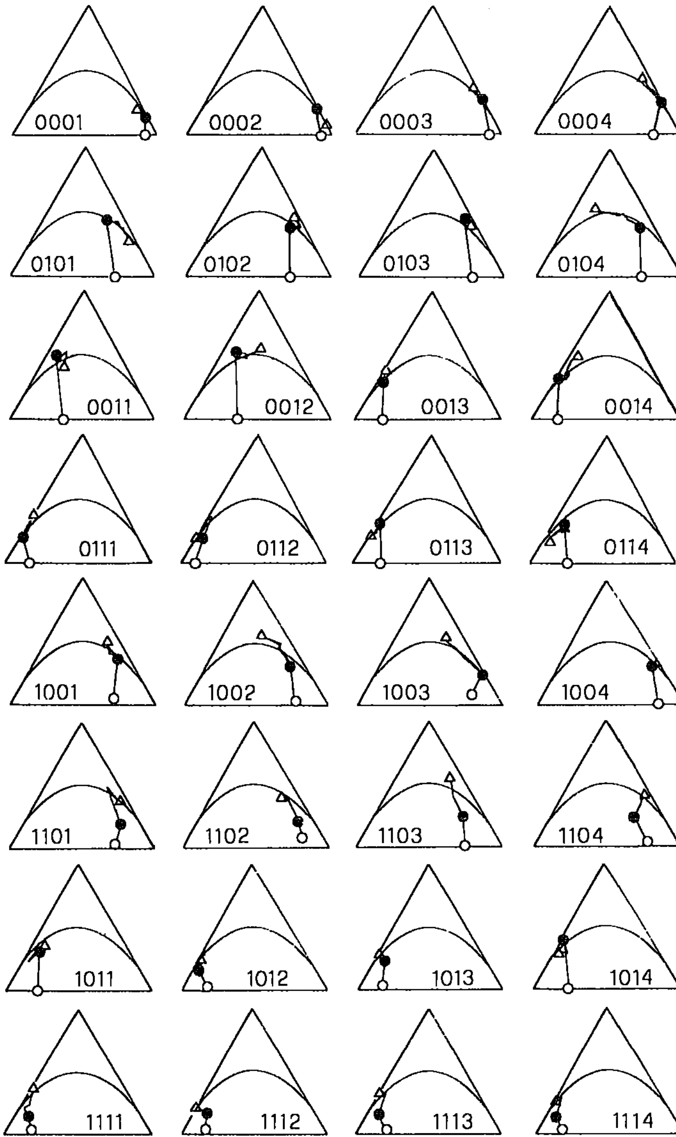

Figure 1. De Finetti diagrams of the *Est*-6 genotype and allele frequency changes over the period of the experiment in the 32 populations. Data for sample 1 are shown with open circles, for sample 2 with closed circles and sample 5 (or 6) with triangles. Genotype frequencies are represented in two dimensions with the right, left and top apices of each triangle corresponding to 100% *SS*, *FF* and *SF* respectively. Each population is denoted by a four-symbol code (a, b, c, d) defined in terms of four factors: (a) temperature (0 = 18°C; 1 = 25°C), (b) treatment (0 = control; 1 = treated), (c) input frequency $p(S)$ (0 = 0·8; 1 = 0·2), (d) replicate number within its particular a, b, c combination (d = 1, 2, 3, 4).

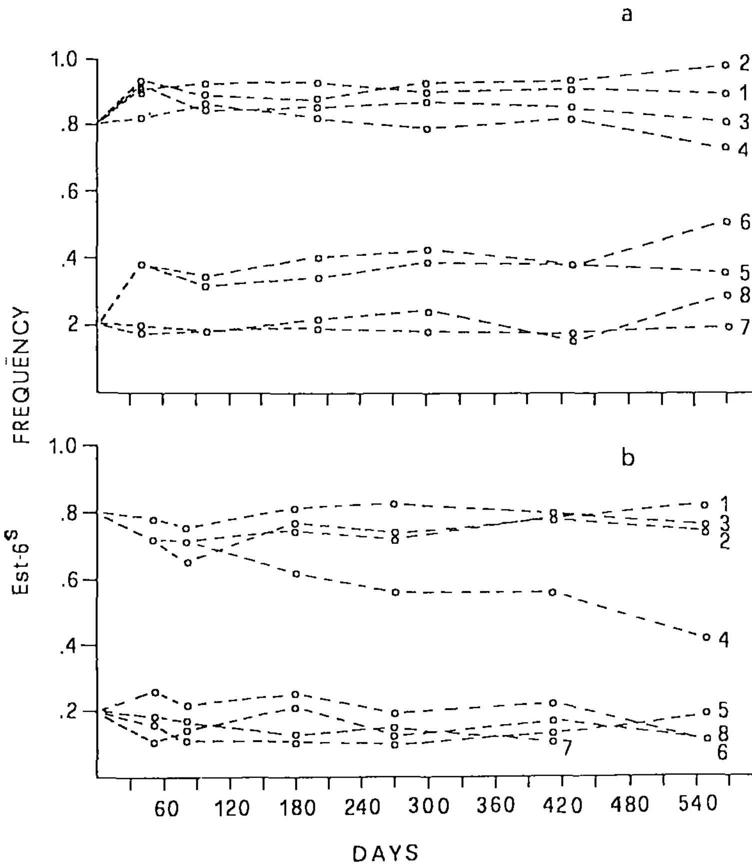

Figure 2. Changes in *Est*-6^S frequencies in control (**a**) and treated (**b**) populations reared at 18°C.

4. Discussion

The results relative to the first sample, when almost no heterozygote is present, confirm the effects of methyl malonate on the *Est-6* genotypic composition reported by Costa *et al* (1985). Here the effects of the treatment are significant only with the input $p(S) = 0{\cdot}8$ at 18°C with a higher apparent fitness for the *Est*-$6^{F/F}$ genotype. In the experiments cited above (Costa *et al* 1985) done at 25°C, when only homozygotes were considered, the *Est*-$6^{F/F}$ genotype also showed the highest fitness but only when the input $p(S)$ was 0·2. The different concentration of the ester (0·03 M) and the different size of the populations (100 females) could account for the observed differences between those and the present results. Fitness reversals in relation to larval density changes have been observed for instance at *ODH*, *MDH-2* and *Est-5* loci in *D. pseudoobsura* (Marinkovic and Ayala 1977).

The results reported here indicate that temperature affects gene frequency, though not significantly, since the frequency of the *Est*-6^S increased in the controls

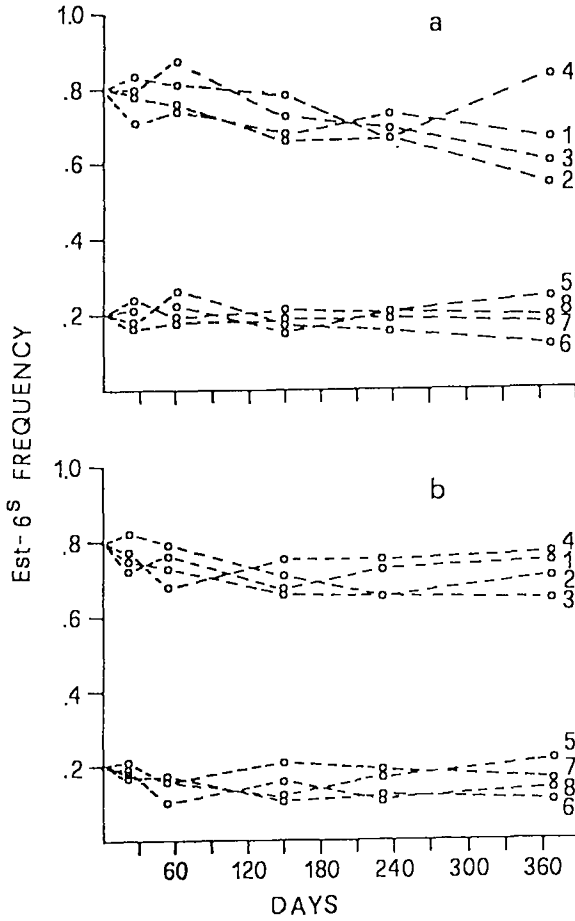

Figure 3. Changes in *Est*-6^S frequencies in control (**a**) and treated (**b**) populations reared at 25°C.

at 18°C and decreased in 3 of 4 population replicates at 25°C when the input $p(S)$ was 0·8. Temperature effects have been previously observed. Oakeshott (1979) and Gilbert and Richmond (1982) in cage population experiments observed *Est-6*

Table 5. Treatment and temperature effects on *Est*-6^S frequency at the input $p(S) = 0·8$ in the first sample. *Est*-6^S increase and decrease are relative to the input values. The P values were obtained applying Fisher's exact test of binomial fit.

		Est-6^S gene frequency increase	decrease	P
18°C	Controls	4	0	0·014
	Treated	0	4	
Controls	18°C	4	0	0·071
	25°C	1	3	

Table 6. Analysis of variance for testing for $Est\text{-}6^S$ frequency changes occurring between the first and second samples.

Source of variability	$p(S)$ = 0·8			$p(S)$ = 0·2		
	var.	d.f.	F	var.	d.f.	F
Total	0·00202	15		0·00081	15	
Between treatments	0·00325	1	1·55 ns	0·00013	1	< 1 ns
Between temperatures	0·00116	1	< 1 ns	0·00005	1	< 1 ns
Interaction treatment × temperatures	0·00069	1	< 1 ns	0·00089	1	< 1 ns
Error (replicates)	0·00210	12		0·00093	12	

frequency changes associated with temperature and the $Est\text{-}6^F$ was seen to decrease at 18°C. Nigro *et al* (1985) also observed appreciable effects of temperature on genotypic output in laboratory populations polymorphic for *Est-6* rare alleles. Interestingly, the direction of the effects of temperature observed by Oakeshott (1979), Gilbert and Richmond (1982) and in the results of the present paper are consistent with the cline in the wild where the $Est\text{-}6^F$ allele shows declining frequencies with decreasing temperatures.

A noteworthy finding is the substantial homogeneity in the results observed between gene frequencies of males and females and the H-W equilibrium observed from the second sample (after about 4 generations) with the exception of the treated (MM) populations at 25°C; in the latter, equilibrium was reached by the third sampling.

Table 7. Analysis of variance for testing for $Est\text{-}6^S$ frequency changes occurring between the second and last samples.

Source of variability	$p(S)$ = 0·8			$p(S)$ = 0·2		
	var.	d.f.	F	var.	d.f.	F
Total	0·01609	15		0·00456	14	
Between treatments	0·01301	1	< 1 ns	0·00737	1	1·98 ns
Between temperatures	0·01081	1	< 1 ns	0·00531	1	1·43 ns
Interaction treatment × temperatures	0·01052	1	< 1 ns	0·01013	1	2·72 ns
Error (replicates)	0·01725	12		0·00372	11	

As for the effects of methyl malonate, it seems likely that they are due to the inhibition of succinate dehydrogenase and the consequent effects on respiratory efficiency caused by one of its cleavage products, the malonate. We suggest that at 18°C the catalytic efficiency of the EST-6^S allozyme could be higher than that of EST-6^F; a greater production of malonate by *Est-6*$^{S/S}$ genotypes with respect to the *Est-6*$^{F/F}$ could be the basis for their greater reduction in fitness.

More intriguing are the results concerning the samples subsequent to the first. No significant differences were found with respect to the results from the first sample at both input frequencies. It seems that, starting from the appearance of heterozygotes, the frequencies established in the early phases of the experiment due to the effects of treatment and temperature are maintained without directional changes.

We cannot exclude the possibility that there is larval competition when the population is made up of only homozygotes and that this is no longer manifested when heterozygotes are present. This would explain our results.

The insignificant variation of frequencies observed after the first sample probably reflects only weak drift effects as populations were maintained with a high number of individuals per cage (3000–5000).

The overall results suggest that, at least in particular laboratory conditions, esterase-6 in ester metabolism can be a target of selection. The lines *Est-6*^{S} and *Est-6*^{F} used here are highly homogeneous for their genetic background, including the *Est-6* regulatory genes (details in Costa *et al* 1985). This supports the hypothesis that the effects of methyl malonate observed in the first sample are due to selection at the *Est-6* locus level. However, it is questionable that the processes taking place in the conditions adopted in the present experiment are actually relevant in natural populations.

Moreover, the results obtained here suggest that a considerable part of the selective phenomena attributed to the *Est-6* polymorphism when several generations were analyzed (MacIntyre and Wright 1966; Yarbrough and Kojima 1967) are to be attributed to other loci possibly in linkage disequilibrium or having epistatic interactions with the *Est-6*. Anxolabehere (1976, 1980) reported evidence of selection in *D. melanogaster* laboratory populations polymorphic at the sepia (3–26·0) locus but he could not exclude effects of other linked or unlinked genes. On the basis of experimental results on the effects of selection on the chromosome segment of *D. melanogaster* included among the glued locus (3–41·4) and the *Est-C* locus (3–49·4) Clegg *et al* (1976) suggested the presence in that region of strong selective effects involving a number of genes and they concluded that the interactions among selected genes were strong. Weak epistatic interactions among loci within the region that spanned *Est-6* (3–36·8) and *Pgm* (3–43·4) were detected successively by Cochrane and Richmond (1980) with an indication that selection did operate in the chromosomal region marked by the *Pgm* locus.

Much more effort is necessary in order to understand the meaning (if any) of the *Est-6* polymorphism and of its peculiar geographic cline. Many data are now available concerning the role of esterase-6 in reproduction and in ester metabolism; the results presented, together with those of Clegg *et al* (1976), Anxolabehere (1976, 1980), Cochrane and Richmond (1980), stress the opportunity for deeper investigations of the possible interactions of *Est-6* with other structural and regulatory loci.

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