ahead of time and stored at −20 °C for as long as 4 weeks, without prolonging defluorescence endpoints beyond those obtained with freshly-prepared mixtures. Longer storage was detrimental, however, as endpoints with older mixtures were delayed by several minutes.

Peroxidase was relatively stable in the dried blood spot sample, as defluorescence endpoints in samples stored at room temperature for up to 1 month were similar to those in samples stored at −20 °C for the same period. Samples stored at −20 °C, furthermore, retained their activity for up to 3 months. Endpoints in dried blood spot samples made from heparinized venous blood were similar to those in samples of capillary blood without anticoagulant when stored as above. Defluorescence endpoints and enzyme activities in the samples from 4 phenylketonuric infants receiving dietary treatment were similar to those of the other samples tested, i.e., endpoints of 6 and 9 min and activities of from 3.1 to 8.2 IU/g hemoglobin. Samples from rats appeared to contain far more enzyme than human blood in tests with the same substrate, in which defluorescence endpoints of the rat blood samples were reached in less than 1 min. The test was modified, therefore, to make it more discriminatory for rat blood peroxidase, by delaying the appearance of endpoints. By doubling the concentration of TPNH in the substrate to 4 mM, the time required for completion of the reaction, i.e., oxidation (defluorescence), was lengthened. Using the more concentrated substrate, activities in dried blood spots from control rats were compared with those in spots from selenium-deficient rats. Defluorescence endpoints in the control groups, in all instances, were 3 min or less, in contrast to those from the deficient rats, which required 20 min. Corresponding whole blood peroxidase activities in healthy, untreated control rats and in selenium-deficient rats were 0.428 and 0.090 IU/mg Hb, respectively (personal communication from Roger A. Sunde) (figure 2).

*Discussion.* The methodology described adds yet another enzyme to the list of those detectable in dried blood spots. The advantages of the new test are the peroxidase's stability in the sample, the small volume of blood required, the convenience of mailing the sample and the assay's rapidity and simplicity. These features should facilitate screening for the defect, the data of which may be used to identify the homozygous phenotype, estimate the defect's frequency and distribution and assess its role in the etiology of hemolytic anemia. Investigators should be aware, however, that partial peroxidase deficiencies are sometimes found in normal infants[3] and should consider the possibility, at the other extreme, that peroxidase deficiencies may be induced following treatment with purified diets deficient in selenium[8].

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## Pollution selection of allozyme polymorphisms in barnacles[1]

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*Summary.* Allozymic variation in proteins encoded by 15 loci was analyzed electrophoretically in 166 individuals of the subtropical acorn barnacle *Balanus amphitrite* from 3 sites varying in pollution levels, situated within 3 km of one another in the Mediterranean Haifa Bay. The 3 sites respectively were a relatively unpolluted marine bay, a petroleum polluted port, and a petrochemically polluted dockyard. Out of the 15 loci tested, 10 exhibited in both 1974 and 1975 statistically significant repetitive trends in allele frequencies in accord with the 3 sites. It is hypothesized that natural selection presumably favours specific alleles in each site, and that in barnacles different allozymic variants function optimally in different polluted environment.

Marine pollution and its effects on living resources[3], including the possible involvement of enzyme polymorphisms such as oxidases and hydrolases, in detoxification of pollutants and sewage has been extensively studied[4–6]. Yet the more general problem of how and to what extent genetic variation found in natural populations is utilized as a basis for adaptive evolution in changing environments is still unanswered[7]. Direct correlation of isozymes with the environment is one promising approach to elucidate this substantive yet controversial problem of evolutionary genetics. We tested the effect of pollution on barnacles in an attempt to assess the role of allozyme polymorphisms in fitness. Our results suggest that allozymic variation is at least partly adaptive in the subtropical acorn barnacle *Balanus amphitrite.*

*Materials and methods.* Acorn barnacles, genus *Balanus,* are nearly cosmopolitan marine crustaceans. Adult barnacles, following larval settlement, are stationary and directly exposed to local environmental variation. We sampled 3 sites varying in pollution levels situated within 3 km of one another in the Mediterranean Haifa Bay (figure 1). Quiet Beach is a regular marine bay, and represents the least polluted site. The Oil Terminal is petroleum-polluted due to discharge from tankers. The Kishon Dockyard, near the Kishon River outlet, is the most polluted site, containing industrial, sewage, and petroleum wastes discharged from dozens of plants, oil refineries, petrochemical and chemical industries. The Kishon site included, according to a 1975 unpublished survey[8], 2 categories of pollutants, a) mineral, amounting to a daily discharge of 6700 $m^3$, highly acidic

Comparison of gene frequencies of chosen alleles at 15 gene loci in *Balanus amphitrite* from 3 localities at Haifa Mediterranean Bay, which differ in pollution levels

| Month and Year | | October 1974, 1 test | | | | | October 1975, 2 tests | | | | | | | | 1974+ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Localities | | Quiet Beach | | Kishon | | | Quiet Beach | | Oil Terminal | | | Kishon | | | 1975 |
| Turbidity[a] | | 6.0 | | 0.25 | | | 6.0 | | 2.1 | | | 0.25 | | | Pooled |
| Locus | Allele | N[b] | P[c] | N | P | Z[d] | N | P | N | P | Z[e] | N | P | Z[d] | Z[f] |
| *Pgi* | *F* | 58 | 0.034 | 60 | 0.133 | 1.93 | 66 | 0.015 | 64 | 0.031 | 0.61 | 62 | 0.097 | 2.05* | 2.80** |
| *Pgm* | *F* | 60 | 0.100 | 58 | 0.517 | 4.92*** | 54 | 0.148 | 42 | 0.310 | 1.90 | 42 | 0.190 | 0.55 | 3.87*** |
| *Me* | $M^+$ | 58 | 0.414 | 58 | 0.466 | 0.56 | 60 | 0.250 | 70 | 0.543 | 3.39*** | 64 | 0.406 | 1.85 | 1.70 |
| *Ao*-1 | *M* | 60 | 0.933 | 58 | 1.000 | 2.00* | 64 | 0.937 | 56 | 0.946 | 0.21 | 56 | 0.929 | 0.20 | - |
| *Ao*-2 | *M* | 48 | 0.875 | 60 | 1.000 | 2.82** | 66 | 0.848 | 68 | 0.956 | 2.10* | 68 | 0.897 | 0.84 | 2.59** |
| *Ao*-3 | *F* | 60 | 0.033 | 58 | 0.121 | 1.73 | 54 | 0.000 | 62 | 0.097 | 2.35* | 54 | 0.130 | 2.74** | 3.20** |
| *Mdh*-2 | *S* | 56 | 0.000 | 60 | 0.033 | 1.37 | 66 | 0.000 | 58 | 0.000 | 0.00 | 56 | 0.018 | 1.09 | 1.74 |
| *Acph*-1 | *M* | 58 | 0.724 | 60 | 0.883 | 2.18* | 64 | 0.750 | 70 | 1.000 | 4.46*** | 68 | 0.941 | 3.06** | 3.71*** |
| *Est*-1 | *F* | 56 | 0.000 | 60 | 0.067 | 1.97* | 58 | 0.000 | 68 | 0.000 | 0.00 | 62 | 0.000 | 0.00 | - |
| *Est*-2 | *F* | 60 | 0.100 | 60 | 0.167 | 1.07 | 66 | 0.258 | 62 | 0.435 | 2.12* | 62 | 0.258 | 0.00 | - |
| *Est*-3 | *M* | 52 | 0.846 | 48 | 0.937 | 1.46 | 58 | 0.672 | 64 | 0.766 | 1.15 | 54 | 0.944 | 3.62*** | 3.59** |
| *Est*-4 | *S* | 60 | 0.000 | 60 | 0.083 | 2.28* | 66 | 0.015 | 70 | 0.000 | 1.03 | 66 | 0.061 | 1.38 | 2.59** |
| *Est*-7 | *M* | 54 | 0.981 | 60 | 1.000 | 1.06 | 36 | 1.000 | 58 | 1.000 | 0.00 | 46 | 1.000 | 0.00 | - |
| *Est*-8 | *M* | 60 | 0.933 | 60 | 0.983 | 1.37 | 66 | 1.000 | 70 | 0.986 | 0.97 | 68 | 1.000 | 0.00 | - |
| *GP* | *M* | 48 | 0.792 | 60 | 0.900 | 1.57 | 42 | 0.786 | 56 | 0.821 | 0.44 | 54 | 0.889 | 1.38 | 2.09* |

[a]Turbidity is measured in m based on the visibility in the water; [b]number of tested genes; [c]P, frequency of tested allele. [d-f]Z, The test statistic comparing proportions in 2 independent samples. $Z^d$ tests the frequencies of the Quiet Beach and the Kishon, in each year separately; $Z^e$ compares those of the Quiet Beach and the Oil Terminal. $Z^f$ was computed by pooling the values of $Z^d$ for 1974 and 1975. Levels of significance = *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

wastes including sulfates, fluorides, chlorides, nitrates, hydrochloric and sulphuric acids, b) organic, amounting to a daily discharge of 7100 $m^3$ basic suspended and dissolved organic materials, including petroleum and its derivatives. Some environmental values for the 3 sites Quiet Beach, Oil Terminal and Kishon, respectively, were: pH 8.2, 8.10, 6.0; turbidity (m) 6.0, 2.4, 0.3; oxygen (ml/l) 5.29, 4.75, 3.16; salinity (‰) 38.7, 38.5, 21.5; organic material (mg/l) 1.0, 1.0, 2.0. In addition, the Oil Terminal differed from the other 2 sites in its high sulfite content (10 ppm as compared to 0.0 in the other 2). The ecological deterioration of the Kishon site is recent. In 1956 all 3 sites included hydrozoans, bryozoans, annelids, ascidians, molluscs and crustaceans among other taxa[9]. In 1975 all these groups were largely extinct in the Kishon due to massive pollution, and only a few species survived, primarily barnacles. In contradistinction most species survived in the Oil Terminal and all survived in Quiet Beach. Thus, species diversity, which is lowest in the Kishon and highest in Quiet Beach, also indicates that the Kishon is the most and Quiet Beach least polluted site.

We compared 166 individuals of *Balanus amphitrite* from the 3 localities for allozymic variation at 15 scorable gene loci in 2 repetitive experiments in October 1974 and October 1975. In order to compare equal age populations in all 3 sites, we immersed 625 $cm^2$ plastic plates 1 m below water surface in May, and removed them in October. Soft tissues of whole animals were homogenized and studied by starch gel electrophoresis[10] for allozymic variation encoded by the following 15 gene loci: phosphoglucose isomerase (*Pgi*); phosphoglucomutase (*Pgm*); malic enzyme (*Me*); aldehyde oxidase, 3 loci (*Ao-1, Ao-2* and *Ao-3*); malate dehydrogenase (*Mdh-2*); acid phosphatase (*Acph-1*); esterase, 6 loci (*Est-1,2,3,4,7,* and *8*), and general protein (*GP*). Figure 2 is a compiled zymogram of the esterases scored in this study.

*Results and discussion.* The number of barnacles per plate averaged in the 1974 and 1975 experiments about 2400 individuals. The largest average barnacle size was in the Kishon (base diameter about 12 mm). Thus surprisingly, the barnacles which lived in the most polluted site were not only among the few surviving species there, but also the largest in size among the barnacles tested. Whether this results from lack of competitors, and/or efficient exploitation of the organic and other pollutants remains unknown. Several consistent trends in allozyme frequencies have been recorded (table). Out of 15 loci tested, 5 (*Mdh-2, Ao-1, Est-1, 7, 8*) showed no consistent differences among the 3 sites. The remaining 10 loci exhibited, in both 1974 and 1975, statistically significant repetitive trends in allele frequencies. a) $Pgi^F$, $Pgm^F$, $Est\text{-}3^M$, $Est\text{-}4^S$, and $GP^M$ increased significantly in the Kishon site as compared with Quiet Beach. b) $Me^{M+}$ and $Est\text{-}2^F$ increased significantly in the Oil Terminal as compared with Quiet Beach. Finally, c) $Ao\text{-}2^M$, $Ao\text{-}3^F$ and $Acph\text{-}1^M$ increased significantly in the Oil Terminal and Kishon as compared with Quiet Beach. Estimates of heterozygosity ($h$) for individual loci increased significantly in Kishon as compared with Quiet Beach in

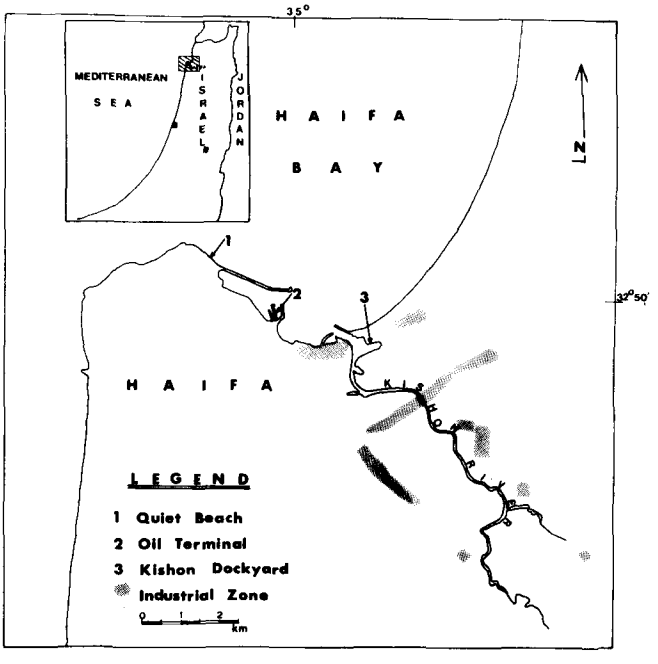


Fig. 1. The 3 testing sites of barnacles in the Haifa Bay, Israel.

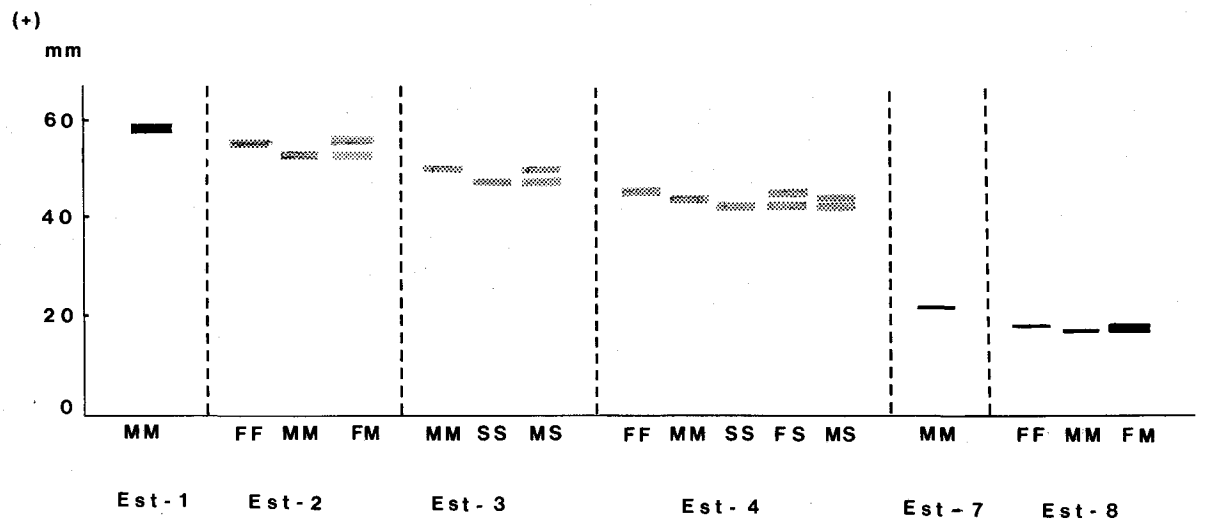


Fig. 2. Variation in esterases of *B. amphitrite*. Genotypes indicated below the origin line.

3 loci (*Pgi, Est-2, Est-4,* $p<0.05$, $<0.01$ and $<0.001$, respectively) and decreased significantly in the Oil Terminal as compared with Quiet Beach in *Me* ($p<0.001$). Mean heterozygosity (*H*), for all 15 loci did not differ significantly between sites (0.084–0.130).

To obtain an approximate measure of the favoured allele in the polluted environment, we assume fitnesses $W_1$, $W_2$, $W_3$ for *AA, Aa* and *aa* respectively, where *a* denotes the favoured allele and *A* all the remaining alleles. The relative fitnesses of *aa* over *AA* ($W_3/W_1$) in the Oil Terminal and the Kishon respectively, as compared with Quiet Beach (= 1.0), are for $Pgm^F$ 18.86, 21.04; $Me^{M+}$ 3.87, 2.19; $Ao\text{-}1^M$ 1.79, 1.90; $Ao\text{-}2^M$ –, 2.92; $Ao\text{-}3^F$ 6.00, 7.00; $Acph\text{-}1^M$ –, 3.95; $Est\text{-}3^M$ 1.28, –; and $GP^M$ 1.18, 2.19. In 6 out of the 8 loci, the relative fitnesses were higher in the Kishon than in the Oil Terminal.

Significant deviations from Hardy-Weinberg expectations due to heterozygote deficiencies were found in 9 out of the 15 loci using the exact test statistic[11]; in Quiet Beach for 6 loci, *Ao-1**, Ao-2**, Ao-3**, Acph-1**, Est-3*** and *GP**;* in Oil Terminal, for 5 loci, *Pgm**, Me**, Ao-3**, Est-3*** and *GP**;* and in the Kishon for 8 loci, *Pgm**, Me**, Ao-1**, Ao-2**, Ao-3**, Acph-1**, Est-1*** and *GP*** (where *, ** denote $p<0.05$ and $p<0.01$, respectively). Altogether 8 of the 9 heterozygote deficiencies were found in the Kishon as against 5 and 6 in each of the other sites. Both the quantitative trends of pollution favoured alleles and the heterozygote deficiencies in the large random mating barnacle populations seem to exclude models of random drift, Wahlund effect, or neutrality and leave selection on single or integrated metabolic phenotypes of transient genetic polymorphisms as the most plausible explanatory model of survival in the polluted environments.

Both the Oil Terminal and the Kishon Dockyard are polluted environments, the latter much more so in terms of heterogeneity and temporal fluctuation of pollutants than the first. Yet in both, selection presumably favours specific but different alleles ($Ao\text{-}2^M$, $Ao\text{-}3^F$ and $Acph\text{-}1^M$ are favoured in both, $Me^{M+}$ and $Est\text{-}2^F$ only in the Oil Terminal, and $Pgi^F$, $Pgm^F$, $Est\text{-}3^M$, $Est\text{-}4^S$ and $GP^M$ only in the Kishon). This is hardly surprising as the 2 polluted sites differ greatly in the kind and degrees of pollutants, as indicated above. The correlation observed between pollution and allozymic variation suggests that in barnacles different allozymic variants function optimally in different polluted environments, and are directly selected accordingly. Noteworthy, out of the 10 loci that changed significantly in the polluted environments, 4 (*Ao-3, Acph-1, Est-3* and *Est-4*) have been also the site of thermal selection in a twin study[12].

No pollution factor may be singled out at present as the specific selective agent of allozyme patterns in barnacles. Several environmental gradients are discernible among the 3 sites (i.e., turbidity and oxygen content), whereas others distinguish either the Kishon (pH, salinity, organic material) or the Oil Terminal (sulfites). Several quantitative allozyme patterns appear to vary in parallel to the environmental gradients ($Ao\text{-}3^F$, $GP^M$), whereas others characterize either the Kishon ($Pgi^F$, *Est-3,4*) or the Oil Terminal ($Me^{M+}$, *Est-2*). However, we currently have 1 repetition only (in the Kishon) showing similar trends, but no repetition in the Oil Terminal (table). Notwithstanding the number of repetitions only direct cause-effect standardized laboratory application of specific pollutants could avoid hypotheses based on post-hoc correlations and unambiguously relate gene frequencies to their selective agent.

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