POPULATION GENETICS OF MARINE PELECYPODS. III. EPISTASIS BETWEEN FUNCTIONALLY RELATED ISOENZYMES OF *MYTILUS EDULIS*[1]

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ABSTRACT

The distribution of interlocus genotypic combinations was examined in *Mytilus edulis* for interdependence between two loci synthesizing functionally related isoenzymes. There is significant dependence between the Leucine Aminopeptidase and Aminopeptidase loci, which we attribute to epistasis, since the magnitude of dependency varies with age. Furthermore, dependency varies in magnitude with position in the intertidal zone from which samples were taken, suggesting that epistasis is a function of the combination of certain non-homologous alleles as well as of the environmental circumstance in which the combinations occur.

EPISTASIS is a concept of gene interaction which has received much theoretical treatment, but little empirical investigation. The interaction of non-homologous alleles may be demonstrated by the presence of stable linkage disequilibrium (CROW and KIMURA 1970), by synergistic effects associated with inbreeding between conspicuous chromosome markers (MUKAI 1969), or by partitioning components of variance (COCKERHAM 1954; KEMPTHORNE 1954, 1955). There are, however, very serious practical limitations to these methods. The number of species for which chromosome mapping is sufficient to use gametic disequilibrium as a criterion for estimating epistasis is severely limited. Analysis of variance may be used to partition the variance of any quantitative character, but the number of loci affecting that character and/or the mapping of those loci are usually unknown. Although epistasis has been demonstrated, (YERMANOS and ALLARD 1961; LEE, COCKERHAM and SMITH 1968; WATKINS and SPANGELO 1968; STUBER and MOLL 1971; and others) and predictions have been made concerning its effects upon the genome, (LEWONTIN 1964a,b; KOJIMA 1969; CROW and KIMURA 1970; FRANKLIN and LEWONTIN 1970), methodo-

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logical restrictions have severely limited the number and scope of studies concerning this important concept of gene action.

Here we attempt to extend interpretations based on single polymorphic loci to consider inter-locus interactions that might be more consistent with the recognized importance of a variable genetic background upon relative fitness of alternate genotypes at a particular locus. We have, without knowledge of linkage groups or gametic frequencies, examined the distribution of di-locus genotypes at the Leucine Aminopeptidase (LAP) and Aminopeptidase (AP) loci in the blue mussel, *Mytilus edulis*, for possible inter-locus dependency (non-random association in samples) between various genotypes. Since these loci synthesize enzyme products of similar catalytic function, we might expect to detect interactions between them more frequently than between loci of more different metabolic function.

MATERIALS AND METHODS

The two loci selected for investigation code for Aminopeptidase (Amino-acyl-dipeptide hydrolase; E.C. 3.4.1.3) and Leucine Aminopeptidase (L-leucyl-peptide hydrolase; E.C. 3.4.1.1.). Methods of tissue preparation, electrophoresis and colorimetric detection of LAP have been previously described (KOEHN and MITTON 1972). Identical methods were used for AP except that detection in starch gels was by the method of SHAW and PRASAD (1970). Both polymorphisms consist of three common and additional rare alleles.

Nomenclature of alleles for both loci follows the conventions established by MILKMAN and BEATY (1970) for Leucine Aminopeptidase of *Mytilus*. That is, alleles at each locus, in order of decreasing electrophoretic mobility are: Lap^f, Ap^f, Lap^m, Ap^m, and Lap^s, Ap^s, etc. For statistical comparison to a HARDY-WEINBERG distribution rare alleles ($P < .01$) were pooled with common alleles of most similar electrophoretic mobility (LI and HOROWITZ 1953). Pooling of alleles was not done in inter-locus analyses.

The data were taken from BALAGOT (1971) as a series of twelve collections at intervals of ten feet in a transect over a gently sloped sand bar at the mouth of the Nissequogue River on Long Island, ranging from extreme high tide to the lowest point of *Mytilus* distribution.

Following electrophoresis, individuals were scored for phenotype at both of *Lap* and *Ap* loci and arranged as a 6×6 contingency table summarizing the observed numbers of each possible inter-locus phenotypic combination. Except for contingency tests upon the total collection, the further subdivision of a sample (e.g., by sex, size, etc.) presents great difficulty because of the enormous sample sizes needed so that all cells of a 6×6 contingency table may be statistically evaluated. This problem increases in magnitude as allele frequencies become less equal to one another. Even with our sample of $n > 1000$, phenotypic combinations involving the rarer third allele are not abundant enough for valid testing. As a consequence, only a subset of the 6×6 contingency table was tested because of these small cell frequencies. That part of the table associated with the rarest allele of each polymorphism was ignored.

If the degree of inter-locus dependence can be shown to change either with microhabitat or with size (age) of the animal, then this would constitute evidence that this dependency is due to some form of epistatic selection. Collections, were, therefore, grouped into samples representing low, middle, and high intertidal microhabitats. In order to test whether or not the pattern of inter-locus dependency of genotype might change with organism size, one would ordinarily perform the analysis (described below) independently upon each size-class. Since our size-classes are by millimeter, over a range of 38–54 mm, a total sample of several thousand individuals would be needed. To avoid this difficulty, samples were subdivided into two groups of "large" and "small" individuals. A contingency table was constructed for the "large" and "small" group, each X^2 value standardized by the total n for the group, and the difference between the standardized contingency X^2's determined. The standardized X^2, a measure of dependence, is termed the

"coefficient of contingency" (SOKAL and SNEATH 1963). Repeated testing in this manner, increasing the cut-off point by one size-class between "large" and "small" individuals for each test, will determine if the degree of dependence between these loci changes as a function of shell size (age). For example, to compute the first point of a regression illustrated in Figure 1, a contingency table consisting of individuals less than or equal to 39 mm ("small") was constructed, the contingency X^2 value determined and divided by the n of that table. The difference between this value and an identically computed value for individuals greater than 39 mm ("large") was then determined. In the next step, "small" would be represented by less than or equal to 40 mm, "large" by greater than 40 mm, and so forth. However, because of the shifting pattern of sample sizes of each size class in the contingency tables, a function with a slightly positive slope will be generated. The expected value of this function, under the null hypothesis of no dependency, is $\frac{N_L - N_S}{N_L N_S} T$ at any point, where N_L, N_S represent sample sizes in "large" and "small" contingency tables, and T represents the degrees of freedom. This can be shown, given that the expected value of X^2 under the null hypothesis is equal to its degrees of freedom:

$$E_{H_0} X_T^2 = T$$

Therefore,

$$E = \frac{X_L^2}{N_L} - \frac{X_S^2}{N_S} = \frac{T}{N_L} - \frac{T}{N_S} = \frac{N_S - N_L}{N_S N_L} T$$

To test whether the observed function is significantly different from the expected function, the distribution of expected regression coefficients was estimated by a randomization test. Using

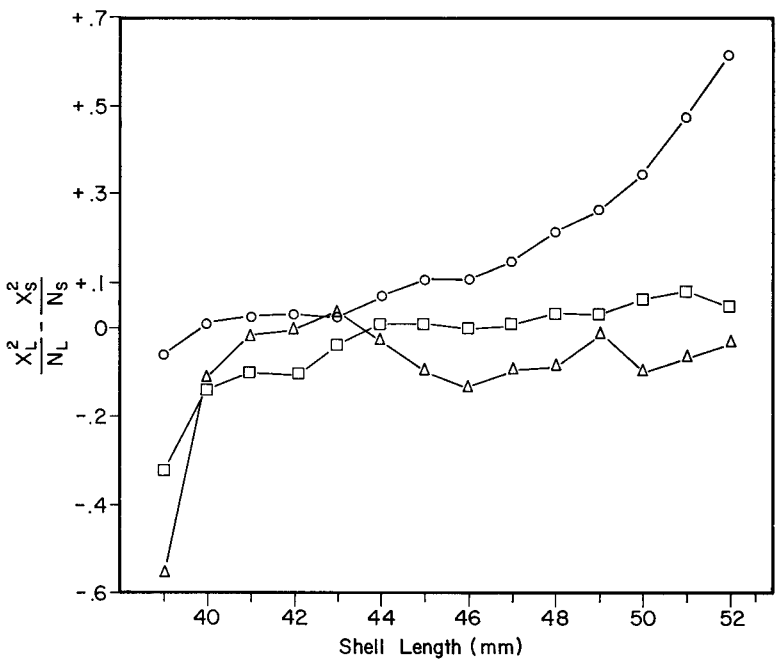

FIGURE 1.—Relationships of differences between coefficients of dependence with increments of shell size for samples from an inter-tidal zone transect where ○ = high-tide, □ = mid-tide, and △ = low tide; χ^2_L/N_L is the coefficient of dependence for the "large" group and χ^2_S/N_S that for the "small" group at each increment.

TABLE 1

Comparison of observed zygotic frequencies and their HARDY-WEINBERG *expectations of the* Lap *and* Ap *loci, including allele frequencies of f(=p), m, (=q), and s (=r) and* χ^2 *values for tests of goodness-of-fit of observed and expected frequencies*

Lap-f	*Lap-fm*	*Lap-m*	*Lap-fs*	*Lap-ms*	*Lap-s*	χ^2
267	380	194	140	99	29	$14.12_{(3)}$, $P < .01$
(250.44)	(412.31)	(169.70)	(141.30)	(116.31)	(19.93)	
	$p = .475 \pm .01$		$q = .391 \pm .01$		$r = .134 \pm .01$	
Ap-f	*Ap-fm*	*Ap-m*	*Ap-fs*	*Ap-ms*	*Ap-s*	
259	274	120	191	124	48	$11.22_{(3)}$, $P < .01$
(237.72)	(308.64)	(100.16)	(198.82)	(129.04)	(41.56)	
	$p = .484 \pm .01$		$q = .314 \pm .01$		$r = .202 \pm .01$	

an IBM 360 computer, 300 iterations of the dependency test described above were run for each microhabitat sample (low, mid, and high tide) using randomly generated shell lengths with the observed genotypes. The observed regression coefficients were then compared to the expected distribution of regression coefficients under the null hypothesis (no relation of inter-locus dependence with shell length) to estimate the probability of observing the relationship of inter-locus dependence with shell size by chance.

RESULTS AND DISCUSSION

For all collections pooled, the observed zygotic frequencies, allele frequencies, and so forth, for the individual *Ap* and *Lap* are given in Table 1. Both loci deviate from a HARDY-WEINBERG expected distribution due to heterozygote deficiencies, an observation consistent with previous studies (MILKMAN and BEATY 1970; KOEHN and MITTON 1972). Deviations from HARDY-WEINBERG at individual loci might contribute to the deviation from expected in di-locus gene combinations. The null hypothesis, that genotypes at the two loci are distributed independently, can be tested without regard to the HARDY-WEINBERG distribution by using a rows-by-columns test of independence where expected frequencies are from row and column totals. A rows-by-columns test of independence upon a contingency table consisting of all collections pooled (Table 2) is significant ($\chi^2 = 16.37_{(4)}$, $P < .005$).

TABLE 2

Observed phenotypic combinations at the Lap *and* Ap *loci and those expected* (in parentheses) *by row and column totals. The distributions are significantly different* ($\chi^2 = 16.47_{(4)}$, $P < .005$)

		Lap— ff	fm	mm
	ff	61	96	49
		(65.58)	(93.36)	(47.06)
Ap—	fm	60	112	56
		(72.58)	(103.33)	(52.09)
	mm	49	34	17
		(31.84)	(45.32)	(22.85)

If the dependence between *Ap* and *Lap* alleles is due to epistatic selection, the difference in dependence between "large" and "small" animals would increase with samples of increasingly older animals, if such selection persists during growth. Changing non-random association of *Ap* and *Lap* alleles with age would be indicated by a significantly changing relationship between size and the coefficient of dependence. This relationship of shell length to magnitude of interlocus dependence was in fact found in one of the three microhabitat samples (Figure 1; Table 3), in which a greater excess of *Ap-m/Lap-f*, and greater deficiencies of *Ap-fm/Lap-f* and *Ap-m/Lap-fm* individuals are found for larger than for smaller individuals. Thus it appears that epistatically-determined differences in fitness must be changing the proportions of genotypes during growth, rather than the interlocus dependency's being due to linkage disequilibrium arising from other causes.

One such alternative cause requires special consideration—namely that a population composed of noninterbreeding component populations can give rise to overall apparent linkage disequilibrium and consequent interlocus dependency among the genotypic combinations. The fact of heterozygote deficiency suggests such a possibility. The evidence against this explanation arises from the following argument: as is demonstrated in the APPENDIX, unless the component populations themselves are in linkage disequilibrium, in order for there to be overall linkage disequilibrium there must be variation in gene frequency among the component populations at *both* loci. In the present case, although there is considerable geographic variation at the *Lap* locus along the Atlantic Coast, frequencies are constant at the *Ap* locus throughout the sampled range from Maine to Virginia ($\chi^2 = 92.544_{(110)}$, $P > .60$; $n = 23$). Thus although the heterozygote deficiency for now remains an enigma (the life cycle essentially rules out inbreeding), the point at the moment is that population heterogeneity apparently cannot account for the interlocus dependency among the genotypic combinations.

In support of selection we also note that the magnitude of interlocus dependency, and the relationship of this dependency to shell size, increase in samples taken from increasingly higher levels above the low tide line (Figure 1), suggesting variation in genotypic fitness over different environments. This would be consistent with BURTON (1968) and YERMANOS and ALLARD (1961), who suggested that the degree of epistasis may vary with genotypic-environmental inter-

TABLE 3

A comparison of observed regression coefficients between size and coefficients of dependency in three microhabitat samples, and those expected by randomization of size on interlocus genotypes (see text for explanation)

Locality	Expected distribution $\bar{b}$	S.D.	Observed b	Probability
High tide	.015	.011	.043	$P < .025$
Mid tide	.014	.008	.022	$P > .16$
Low tide	.010	.009	.011	$P > .46$

actions. Thus epistasis between the two peptidase loci in Mytilus appears to depend both on the particular non-alleles and the environmental circumstance in which particular genotypic combinations occur.

Epistasis between the products of single genes makes investigation of the nature of the interlocus molecular interaction at least theoretically possible. There are no obvious characteristics (e.g., members of adjacent steps in a biosynthetic pathway) of the two peptidases that would necessarily lead to *a priori* predictions of epistasis between them, other than that they are both similar catabolic enzymes. We must also consider the notion that epistasis is not necessarily between the *Lap* and *Ap* loci, but involves other loci closely linked to them. Quite obviously, our interpretations here would be less equivocable with inheritance data from individual matings. We are not so interested in attempting, by a process of deductive reasoning, to unequivocably establish the role of epistasis, but to point out that the inter-locus genotypes are not necessarily distributed randomly in natural populations and that the approach we have taken may be valuable in elucidating patterns of selection.

We suggested in an earlier paper (KOEHN and MITTON 1972) that allelic variants at the *Lap* locus of *Mytilus edulis* were maintained by selection as some component of the physical environment. This conclusion was based on parallel patterns of genic variability and changes in genetic composition over an environmental gradient at the *Lap* loci of *Mytilus edulis* and *Modiolus demissus*. Parallel changes at single loci in different organisms of similar habitat suggested a minor role for genetic background in the evolution of the observed patterns of heterogeneity at this locus. Results described here are consistent with our earlier interpretations, if we assume that a given locus (i.e., *Lap*) may be influenced by various forms of natural selection, so that selection influences the *Lap* phenotypes, *per se*, but additional selective responses occur that are related to a multi-locus phenotype. This would further lead us to suggest what must be entirely obvious to evolutionists, that there is a genetic synergism in an organism's response to natural selection, and that unique single locus fitness estimates are unrealistic. It would, therefore, be desirable to examine numerous polymorphic loci to see where epistatic interactions might be discovered and whether these would be restricted to functionally related enzymes.

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APPENDIX

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In the body of this paper it was pointed out that a mixture of non interbreeding populations, each one in linkage equilibrium, can give overall linkage disequilibrium. However, such an explanation was ruled out in this case, on the grounds that for such an effect to occur, allele frequencies must vary among component populations at *both* loci. The object of this APPENDIX is to prove this latter assertion, and, also, incidentally, to point out some of the properties of linkage equilibrium in non interbreeding mixtures.

For simplicity, the following treatment will be in terms of gametic frequencies and then, by a simple extension, genotype frequencies will be considered.

CAVALLI-SFORZA and BODMER (1971, p. 69) point out that in a non interbreeding mixture

composed of just two component populations, the linkage disequilibrium will be as follows:

$$D = f_1 f_2 (p_1 - p_2)(r_1 - r_2) \quad (1)$$

where:

$D \equiv$ conventional measure of linkage disequilibrium

$f_1, f_2 \equiv$ the relative abundance of the two component populations 1 and 2, respectively; $f_1 + f_2 = 1$

$p_1, p_2 \equiv$ frequency of the same allele at one locus in component populations 1 and 2, respectively

$r_1, r_2 \equiv$ frequency of the same allele at the other locus in component populations 1 and 2, respectively

This clearly shows that, for two components, in order for $D \neq 0$, $p_1 \neq p_2$ and $r_1 \neq r_2$ (as will be seen presently this assumes the mean D between the components is zero). This result can be readily generalized to a mixture composed of n component populations.

Consider two bi-allelic loci where the k^{th} component population has frequency f_k, and the allele frequencies are p_k at the first locus and r_k at the second.

APPENDIX TABLE 1

The gametic contingency tables for the k^{th} component population and for the total population

k^{th} component with frequency f_k

	A	a	
B	$p_k r_k + D_k$		p_k
b			$1-p_k$
	r_k	$1-r_k$	

Total Population

	A	a	
B	$\sum_{k=1}^{n} f_k (p_k r_k + D_k)$		$\bar{p}$
b			$1-\bar{p}$
	$\bar{r}$	$1-\bar{r}$	

Considering just one cell of the table is sufficient, since the linkage disequilibrium, D, in the total population is simply the difference between "observed" and "expected" in any cell.

Thus

$$D = \sum_{k=1}^{n} f_k (p_k r_k + D_k) - \bar{p}\bar{r} \quad (2)$$

It can be easily shown that

$$\bar{p} = \Sigma f_k p_k$$
$$\bar{r} = \Sigma f_k r_k$$

Therefore (2) becomes

$$D = \Sigma f_k D + \Sigma f_k p_k r_k - \Sigma f_k p_k \Sigma f_k r_k$$

The last two terms constitute the covariance of p_k, r_k; so we have

$$D = \overline{D} + \text{Cov}\ (p_k, r_k) \tag{3}$$

If all components are in linkage equilibrium, or even if their mean is, then $\overline{D} = 0$ and (2) becomes

$$D = \text{Cov}\ (p_k, r_k) \tag{4}$$

A more useful form of (4) can be had by writing it in terms of the correlation coefficient r, rather than the covariance. This gives

$$D = r\ \sqrt{\sigma^2{}_p \sigma^2{}_k}$$

where

$$\begin{aligned} \sigma^2{}_p &\equiv \text{variance of } p_k \\ \sigma^2{}_r &\equiv \text{variance if } r_k \end{aligned} \tag{5}$$

Thus we have the generalization that if $\overline{D} = 0$ a necessary condition for $D \neq 0$ is that $\sigma^2{}_p$, $\sigma^2{}_r > 0$, i.e., allele frequencies must vary at both loci, otherwise $D = 0$.

Some additional aspects of (5) are worth noting. It will be recalled (Li 1955, p. 297) that the deficiency of heterozygotes among the genotypes of the loci taken separately are related to the variance of p_k and r_k as follows:

$$\begin{aligned} d_p &= 2\sigma^2{}_p \\ d_r &= 2\sigma^2{}_r \end{aligned}$$

where d_p, $d_r \equiv$ the deficiency of heterozygotes at the "p" locus and the "r" locus respectively.

Substituting in (5) gives

$$D = \frac{r}{2}\ \sqrt{d_p d_r} \tag{6}$$

When the mixture contains just two components ($k = 1, 2$) as in the codfish mixtures described by Sick (1965) then (6) becomes

$$D = \tfrac{1}{2}\ \sqrt{d_p d_r} \tag{7}$$

which can be easily shown to be equivalent to (1) given by Cavalli-Sforza and Bodmer (1971).

To calculate gametes in a nonbreeding mixture may appear not to make sense biologically, unless one were considering a hybrid swarm in which mating *did* occur, in which case (7) or (3) demonstrate the long-recognized fact that hybridization generates linkage disequilibrium from which considerable time could be required to recover. Otherwise the gametes considered above may be regarded as those which produced the genotypes which are now mixing but not mating. In practice these gametes could be calculated from the genotypes when sufficient information is available

In the case discussed in the body of the paper there is not sufficient information to make such a calculation from genotypes, and the lack of independence was in fact demonstrated in a genotypic contingency table. For such a table with dimensions greater than 2×2, the convenient measure of dependence, D, is no longer available. However, it is a simple matter to prove the assertion that if the genotype frequencies at one locus are invariant among the components, then the entire table will exhibit independence—provided, of course, that there is no "average dependence" (analogous to $\overline{D}$) among the components themselves.

As before let f_k be the frequency of the k^{th} component population. Further, let Q_{ik} be the frequency of the i^{th} genotype produced by the first locus, and P_{jk} be that of the j^{th} genotype produced by the second locus in the k^{th} component population.

We now calculate the "observed" minus "expected" in the $i^{th}\ j^{th}$ cell of the table, D_{ij}, for the total population. In analogous fashion to the previous result we have:

$$D_{ij} = r_{ij}\ \sqrt{\sigma^2{}_{Qi}\ \sigma_{Pj}} \qquad (\text{assuming } \overline{D}_{ij} = 0)$$

If, for instance, the Q_i genotype is invariant over all component populations, then $D_{ij} = 0$.

If all of the genotype frequencies at the first locus are invariant ($\sigma^2_{Qi} = 0$ for all i) then D_{ij} in all cells will be zero and the table will exhibit independence.

If the components themselves exhibit dependence, then this of course could account for the dependence in the total population even with invariant genotype frequencies. However, without further field data, such a consideration is quite useless, since one is now forced to inquire concerning the origin of this dependence (hypothetical) in the component populations (hypothetical), and clearly this exercise has no end.

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