Allozyme and RFLP Heterozygosities as Correlates of Growth Rate in the Scallop Placopecten magellanicus: A Test of the Associative Overdominance Hypothesis

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ABSTRACT

Several studies have reported positive correlations between the degree of enzyme heterozygosity and fitness-related traits. Notable among these are the correlations between heterozygosity and growth rate in marine bivalves. Whether the correlation is the result of intrinsic functional differences between enzyme variants at the electrophoretic loci scored or arises from non-random genotypic associations between these loci and others segregating for deleterious recessive genes (the associative overdominance hypothesis) is a matter of continuing debate. A prediction of the associative overdominance hypothesis, not shared by explanations that treat the enzyme loci as causative agents of the correlation, is that the correlation is not specific to the type of genetic marker used. We have tested this prediction by scoring heterozygosity at single locus nuclear restriction fragment length polymorphisms (RFLPs) in a cohort of juvenile scallops (Placopecten magellanicus) in which growth rate was known to be positively correlated with an individual’s degree of allozyme heterozygosity. A total of 222 individuals were scored for their genotypes at seven allozyme loci, two nonspecific protein loci of unknown function and eight nuclear RFLPs detected by anonymous cDNA probes. In contrast to the enzyme loci, no correlation was observed between growth rate and the degree of heterozygosity at the DNA markers. Furthermore, there was no relationship between the magnitude of heterozygote deficiency at a locus and its effect on the correlation. The differences observed between the effects of allozyme and RFLP heterozygosity on growth rate provide evidence against the associative overdominance hypothesis, but a strong case against this explanation must await corroboration from similar studies in different species.

Although a correlation between an individual’s degree of heterozygosity and fitness is predicted under a wide range of population genetics models (Ohta 1971; Berger 1976; Turelli and Ginzburg 1983; Charlesworth 1991), its demonstration in natural populations has met with mixed success [reviewed by Zouros and Foltz (1987); see also recent studies by Houle (1989), Gaffney (1990), and Booth et al. (1990)]. Where a correlation is found, as is often the case with allozyme heterozygosity and growth rate in cohorts of marine bivalves (Singh and Zouros 1978; Zouros et al. 1980; Fujio 1982; Koehn and Gaffney 1984; Koehn et al. 1988; Zouros et al. 1988; Gentili and Beaumont 1988; Alvarez et al. 1989), it has proven difficult to unambiguously distinguish between hypotheses that treat the enzyme loci as causative agents of the correlation or as neutral markers of genetic conditions responsible for the correlation (Zouros 1990). This dilemma is clearly evident in the most comprehensive study to date in which fifteen allozyme loci were scored in over 1,900 individuals of the coat clam, Mulinia lateralis by Koehn et al. (1988). To distinguish between the two types of hypotheses Koehn et al. (1988) examined whether there was a connection between the metabolic function of the enzyme and its effect on the correlation. They observed that enzymes situated within, or feeding into, glycolysis or involved in the catabolism of proteins exerted a stronger effect on the correlation between heterozygosity and growth than enzymes functioning in other metabolic pathways. This observation provides evidence favoring the selective importance of polymorphisms at specific loci for it is difficult for hypotheses that treat the enzyme loci as neutral to account for differences related to the functional properties of their products. However, in a further examination of the same data Gaffney et al. (1990) observed a correlation between the magnitude of the effect of a locus on the correlation and the locus’ fixation index (expressed as the deficiency in the number of heterozygotes in the sample). This observation cannot easily be explained by postulating a selective role for electrophoretic variants at these loci, but is predicted from the hypothesis that the allozyme loci are linked to deleterious genetic conditions affecting growth rate.

Several conditions may lead to an apparent heterozygote advantage at a scored locus (these are listed and discussed briefly in Zouros and Mallet 1989). Most im-

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important among these are genotypic correlations among alleles at the scored locus with those at a second unscored locus. The unscored locus can itself be heterotic, but would most likely segregate for deleterious genes that are partially or fully recessive (Crow and Simmons 1983). We use the term associative overdominance to refer to the phenotypic outcome of these correlations. The term was originally coined by Frydenberg (1963) and has been widely used since (e.g., Ohta 1971), mainly because it emphasizes the distinction between true overdominance and apparent overdominance due to associations with other loci. Genotypic correlations leading to associative overdominance can arise from a variety of reasons [see Houle (1989) for a comprehensive discussion]. These can be classified into those causing linkage disequilibrium in the population's gametic pool and those producing genotypic correlations over a single generation due to non-random union of gametes (Zouros 1993). The search for evidence favoring the associative overdominance hypothesis has followed this distinction. Analysis of electrophoretic data for linkage disequilibria in organisms exhibiting heterozygosity-growth correlations has produced generally negative results (Ahmad and Hedrick 1985; Mallet and Zouros 1987), which is not surprising given the large recombination distances between even the most closely linked allozyme loci in these species (Foltz 1986; A.R. Beaumont, personal communication). Attempts to implicate inbreeding, and thus associative overdominance, in studies of heterozygosity-fitness correlations have been more successful as most studies that have reported positive correlations have indeed been characterized by significant heterozygote deficiencies (Zouros 1987). Further evidence supporting associative overdominance comes from the observation that locus-specific effects on the correlation are related to locus-specific heterozygote deficiencies (Gaffney et al. 1990). Associative overdominance makes a number of additional predictions which are, in theory, testable. For example, it predicts that at a marker locus there should exist an inverse relationship between the fitness depression of a homozygous genotype and its allele frequency, and that among marker loci there should be an inverse relationship between the effect of a locus on the overall correlation and its expected heterozygosity (Zouros 1993). Unfortunately, both of these relationships are also predicted by models of balancing selection acting at the scored locus (Smouse 1986) or by undetected null alleles (Pogson 1991).

A critical evaluation of the hypothesis that allozymes are directly responsible for the heterozygosity-fitness correlation rather than markers of undetected selected factors requires the development of tests in which the competing hypotheses make opposing predictions. Here we report the application of one such test. We developed a specific set of cDNA probes that detect single locus polymorphisms at sites closely linked to transcribed regions of the genome and tested for the correlation between heterozygosity at these markers and shell height in a cohort of the deep-sea scallop, Placopecten magellanicus, in which allozyme heterozygosity was already known to correlate positively with growth rate (Zouros et al. 1992). We reasoned that under the associative overdominance hypothesis the DNA polymorphisms should have similar effects (and, therefore, produce a similar correlation) as the allozyme loci, since both types of polymorphisms merely serve to mark chromosomal regions harboring deleterious recessive genes. In contrast, under the hypothesis that allozymic heterozygosity is directly favored by selection, heterozygosity scored at the DNA level should not correlate with growth rate. Our results agree with the latter prediction and thus provide evidence against the associative overdominance explanation of the heterozygosity-fitness correlation.

MATERIALS AND METHODS

Animals: A cohort of the deep-sea scallop, P. magellanicus (Gmelin), was collected as spat in September 1987 from a natural population in Paspamquoddy Bay, New Brunswick, Canada, as described in Zouros et al. (1992). After 9 months juveniles were moved to Sambro, Nova Scotia, Canada, and reared in pearl nets suspended 2–4 m above the bottom. In July 1989 a random sample of individuals was brought into the laboratory and held in ambient, running seawater tanks. Over a 4-week period, the adductor muscles were excised from a total of 244 individuals. Approximately two-thirds of the adductor muscle was used immediately for the purification of mitochondrial DNA, and the remainder was stored at −80°C until used for scoring allozymes and the extraction of total DNA (see below). The shell height of the left valve was measured to the nearest 0.01 mm with Mitutoyo digital calipers.

Protein electrophoresis: The electrophoretic procedure used is outlined in Zouros et al. (1992). Six enzyme and two general (nonspecific) protein loci were scored on a 155 mm Tris-HCl, 43 mM citrate, pH 7.0, buffer system: phosphoglucone isomerase (Pgi; EC 5.3.1.9), 6-phosphogluconate dehydrogenase (PgD; EC 1.1.1.44), glutamate-oxaloacetate transaminase (Got; EC 2.6.1.1), adenylate kinase (Adk; EC 2.7.4.3), general protein 1 (Ngp1) and general protein 2 (Ngp2). Three enzyme loci were scored on a 100 mM Tris-HCl, 100 mM borate, 20 mM EDTA, pH 8.5, buffer system: phosphoglucomutase (PgM; EC 2.7.5.1), octopine dehydrogenase (Odh; EC 1.5.1.11), and phosphomannose isomerase (Pmi; EC 5.3.1.8). Allozymes were visualized using standard staining assays. Non-specific proteins were stained with 0.2% amido black in methanol:acetic acid/water (5:4:1). The scoring of alleles followed Foltz and Zouros (1984) and Völkerberg and Zouros (1989) except for Adk and the two nonspecific proteins which were not scored in these previous studies.

DNA extractions: Approximately 0.3 g of adductor muscle was ground in liquid nitrogen and added to 700 µl of lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA, 0.8% sodium dodecyl sulfate (SDS), pH 8.3, containing 200 µg proteinase K). After digesting the tissue overnight at 55°C, total DNA was extracted twice with 1 volume of phenol, once with 1 volume of phenol:chloroform (1:1) and once with 1 volume of chloroform in a 9.5 ml SST tube. Following precipitation of the DNA with 1 volume of isopropanol, the samples were centrifuged at 14,000 rpm for 25 min, washed with 70% EtOH, and
resuspended in 100 µl TE, pH 8.0. DNA concentrations were determined by measuring absorbance in duplicate at 260 nm on a Beckman DU-64 UV/Visual spectrophotometer and checked visually by running a 5-µl aliquot on a 0.8% agarose gel and staining with ethidium bromide.

**Probe isolation:** A cDNA library was commercially prepared in the phagemid vector Lambda Bluestrip from poly(A+) RNA extracted from adult scallop adductor muscle (Clontech). Amplified and unamplified forms of the library were plated out at low density (<40 plaques/plate) and color selected for the presence of inserts. Individual plaques were picked with a large bore pipette tip and transferred to 100 µl SM buffer (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4). After storage for a minimum of 24 h at 4°, an aliquot of phage stock was frozen and thawed twice, and the cDNA insert amplified from the phagemid DNA by the polymerase chain reaction (PCR) using primers that flanked the EcoRI cloning site. The PCR reaction mix contained 10 mM Tris-HCl (pH 8.30 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dATP, dCTP, dGTP, and dTTP, 0.4 µM SR primer (5′-TCTAGAAGCTATGGAATCT-3′), 0.4 µM KS primer (5′-CGAAGTCGACGGTATCG-3′), 5 µl template DNA and 1 unit of Taq polymerase in a final volume of 50 µl. After 30 cycles of denaturation at 94° (1 min), primer annealing at 45° (1 min) and extension at 72° (2.5 min) on a Perkin-Elmer Cetus DNA Thermal Cycler, 3-µl aliquots of the reactions were run out on 0.8% agarose gels in 1 X TBE, pH 8.3, and the amplified cDNA inserts visualized with ethidium bromide.

**Detection of RFLP polymorphism:** A random sample of cDNA fragments ranging in size from 0.5 to 1.2 kb were selected as probes to screen for restriction fragment length polymorphisms (RFLPs) by Southern blot analysis. Screening blots were pre-hybridized with PM DIG 1-dUTP into the template at a concentration of 2× SSC, 0.2% SDS, and 200 µg/ml RNA (from Torula yeast) at 42° C. Hybridizations were carried out overnight at 42° in 55% formamide, 5× SSPE, 0.5× Denhardt’s solution, 0.5% SDS, and 200 µg/ml RNA (from Torula yeast) at 42° C. Hybridizations were carried out overnight at 42° in 55% formamide, 5× SSPE, 0.5% SDS, and 200 µg/ml RNA (from Torula yeast) at 42° C. Hybridizations were carried out overnight at 42° in 55% formamide, 5× SSPE, 0.5% SDS, and 200 µg/ml RNA. Blots were washed twice for 20 min in 2X SSC, 0.2% SDS at room temperature. The chemiluminescent assay of Tizard et al. (1990) was used to detect restriction fragments. After application of the chemiluminescent substrate (0.125 mM AMPFD from Tropix Inc.), blots were exposed to Kodak X-OMAT X-ray film for 3–5 h.

**Scoring of DNA polymorphism:** DNA samples (10 µg) from each individual were digested with three restriction enzymes (HaeIII, HpaII and RsaI), electrophoresed on 0.8% agarose gels, transferred to nylon membranes and probed as described above using the cDNA probes listed in Table 1. Probes PM168, PM238, PM322, PM360, and PM364 were hybridized against DNA digested with HaeIII, probes PM182 and PM318 to RsaI digests, and probe PM388 to DNA digested with HpaII. Restriction fragment sizes were estimated by unweighted linear regression relative to the positions of DNA size standards (BRL 1-kb ladder) run in three lanes of each gel. Two reference individuals were also included on all gels. For the accurate reading of genotypes at the highly polymorphic DNA loci detected by probes PM168, PM322 and PM388, restriction fragment sizes were measured in triplicate and assigned into fixed size “bins” following the protocol of Budowle et al. (1991). The error in sizing alleles at all loci was estimated to be less than 1.0% of the DNA fragment’s length. At each locus, alleles were assigned to bins that clearly exceeded this measurement error and spanned the entire size range of restriction fragments.

**RESULTS**

A total of 222 individuals were scored for their genotypes at 17 loci as listed in Table 1. Nine of the loci encoded for proteins and these polymorphisms were scored through the differences in electrophoretic mobilities of their native protein products. Seven of these loci represent typical allozyme polymorphisms scored in most electrophoretic surveys. The other two loci code for abundant proteins that apparently lack enzymatic activity. The remaining eight loci were RFLPs. Two typical examples of this type of polymorphism are shown in Figure 1. In Figure 1A, total DNA from seven unrelated individuals was digested with HaeIII and probed with cDNA probe PM322. Nine fragments can be seen ranging in size from approximately 5.4 to 11.6 kb. When the same DNA samples were digested with TaqI and probed with PM322, the size range of the fragments changed to 4.1 to 10.3 kb, but the size difference between allelic bands remained the same (not shown). This suggests that PM322 detects a single-locus variable number of tandem repeats (VNTR), i.e., a polymorphism due to the existence of arrays of tandem repeats, with the number of repeats varying among allelic arrays. The fact that we have not observed a three-handed individual suggests that the restriction enymes cut in single-copy DNA flanking the section of the gene that hybridizes to the probe, and that the array of repeats is closely linked to the transcriptional unit. The segregation of these arrays was tested in two pair matings [one in which both parents were scored and one in which only the mother was available for scoring (data not shown)] and in both cases was found to be completely consistent with Mendelian expectations. Four other cDNA probes produced polymorphisms with properties similar to PM322. We refer to these five loci as VNTR polymorphisms.

In Figure 1B the same DNA samples were digested with RsaI and hybridized against cDNA probe PM182. This probe did not detect a comparable polymorphism when the DNA was cut with any other enzyme tested (Pst, HpaII, Sau3A or HaeIII). The dependence of the polymorphism on a specific restriction enzyme suggests that it most likely is caused by the presence or absence

Allozyme and RFLP Heterozygosity 223
Again, we failed to see three-banded individuals at these loci. As revealed by probe PM182, VNTR polymorphisms were suggested that the variable restriction sites are located outside the coding region that hybridizes with the probe. Because each probe used in the study was used to score only one single-locus polymorphism, the designation of the probe is also used to designate the polymorphic locus detected by that probe. A more detailed description of these polymorphisms appears in Pogson (1994).

At eight loci the mean shell height of heterozygotes was larger than the mean shell height of homozygotes. Partial F tests indicated that two positive differences (PM238 and Adk) and one negative difference (PM322) were statistically significant. The sign test and the sequential Bonferroni test (Rice 1989) suggest that there is no overall trend for heterozygote means to exceed homozygote means. The question of whether there is a systematic difference in shell height between homozygotes and heterozygotes for different classes of loci can be approached in several ways. One follows from the original intention of the study and is based on the distinction between enzyme-coding loci and loci that do not code for enzymes. The justification of this classification is that all previous studies that have sought to correlate phenotypic traits with the degree of individual heterozygosity have exclusively used allozyme variation. According to this criterion the seventeen loci in Table 1 are classified into two groups, one of seven (enzyme loci) and one of ten (two nonspecific proteins and eight RFLPs). Two a posteriori classifications also merit attention. One is based on whether the polymorphism is scored from a protein product or not. According to this criterion, the loci are divided into two mutually exclusive groups and the two regressions were compared to each other and to the overall regression.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Class</th>
<th>$N$</th>
<th>Homozygotes $\pm SE$</th>
<th>Heterozygotes $\pm SE$</th>
<th>Rank order</th>
<th>No. of alleles</th>
<th>$H_o$</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgm</td>
<td>E1</td>
<td>133</td>
<td>53.85 ± 0.51</td>
<td>89</td>
<td>55.04 ± 0.63</td>
<td>3</td>
<td>5</td>
<td>0.401</td>
</tr>
<tr>
<td>Pgi</td>
<td>E1</td>
<td>202</td>
<td>54.20 ± 0.41</td>
<td>20</td>
<td>55.62 ± 1.38</td>
<td>7</td>
<td>3</td>
<td>0.009</td>
</tr>
<tr>
<td>Odh</td>
<td>E1</td>
<td>104</td>
<td>53.55 ± 0.59</td>
<td>118</td>
<td>55.02 ± 0.52</td>
<td>4</td>
<td>5</td>
<td>0.532</td>
</tr>
<tr>
<td>Pmi</td>
<td>E1</td>
<td>137</td>
<td>54.95 ± 0.46</td>
<td>85</td>
<td>53.33 ± 0.71</td>
<td>16</td>
<td>3</td>
<td>0.383</td>
</tr>
<tr>
<td>Got</td>
<td>E1</td>
<td>100</td>
<td>53.72 ± 0.61</td>
<td>122</td>
<td>54.83 ± 0.52</td>
<td>5</td>
<td>5</td>
<td>0.550</td>
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<tr>
<td>Adk</td>
<td>E2</td>
<td>117</td>
<td>53.79 ± 0.56</td>
<td>105</td>
<td>54.93 ± 0.35</td>
<td>2</td>
<td>4</td>
<td>0.473</td>
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<tr>
<td>Pgcl</td>
<td>E2</td>
<td>168</td>
<td>54.34 ± 0.47</td>
<td>54</td>
<td>54.30 ± 0.69</td>
<td>9</td>
<td>4</td>
<td>0.243</td>
</tr>
<tr>
<td>Nsp1</td>
<td>UF</td>
<td>213</td>
<td>54.45 ± 0.40</td>
<td>9</td>
<td>51.58 ± 1.82</td>
<td>15</td>
<td>2</td>
<td>0.041</td>
</tr>
<tr>
<td>Nsp2</td>
<td>UF</td>
<td>120</td>
<td>54.35 ± 0.56</td>
<td>102</td>
<td>54.31 ± 0.56</td>
<td>12</td>
<td>3</td>
<td>0.459</td>
</tr>
<tr>
<td>PM182</td>
<td>RS</td>
<td>168</td>
<td>54.35 ± 0.46</td>
<td>54</td>
<td>54.28 ± 0.77</td>
<td>14</td>
<td>3</td>
<td>0.243</td>
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<tr>
<td>PM238</td>
<td>RS</td>
<td>196</td>
<td>53.98 ± 0.43</td>
<td>26</td>
<td>57.01 ± 0.76</td>
<td>4</td>
<td>1</td>
<td>0.117</td>
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<tr>
<td>PM364</td>
<td>RS</td>
<td>114</td>
<td>54.88 ± 0.55</td>
<td>108</td>
<td>53.96 ± 0.57</td>
<td>13</td>
<td>5</td>
<td>0.487</td>
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<tr>
<td>PM168</td>
<td>VNTR</td>
<td>48</td>
<td>54.50 ± 0.91</td>
<td>174</td>
<td>54.28 ± 0.44</td>
<td>10</td>
<td>19</td>
<td>0.784</td>
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<tr>
<td>PM318</td>
<td>VNTR</td>
<td>84</td>
<td>54.14 ± 0.61</td>
<td>138</td>
<td>54.45 ± 0.52</td>
<td>8</td>
<td>5</td>
<td>0.622</td>
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<tr>
<td>PM322</td>
<td>VNTR</td>
<td>15</td>
<td>57.47 ± 1.48</td>
<td>207</td>
<td>54.10 ± 0.41</td>
<td>17</td>
<td>41</td>
<td>0.939</td>
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<tr>
<td>PM360</td>
<td>VNTR</td>
<td>131</td>
<td>54.49 ± 0.50</td>
<td>91</td>
<td>54.10 ± 0.64</td>
<td>11</td>
<td>6</td>
<td>0.410</td>
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<tr>
<td>PM388</td>
<td>VNTR</td>
<td>23</td>
<td>54.06 ± 1.32</td>
<td>199</td>
<td>54.36 ± 0.42</td>
<td>6</td>
<td>28</td>
<td>0.896</td>
</tr>
</tbody>
</table>

TABLE 1

Single locus statistics

$E_1$: protein catabolic, glycolytic or preglycolytic (cf. KOHN et al. 1988); $E_2$: not $E_1$: UF; unknown function. $H_o = \text{observed heterozygosity}$, $D = (H_o - H_e)/H_e$. $H_e = \text{expected heterozygosity}$.

FIGURE 1.—Single-locus restriction fragment length polymorphisms revealed by cDNA probes. (A) VNTR polymorphism revealed by probe PM322. (B) RS polymorphism revealed by probe PM182.

of a restriction site. Two other probes detected polymorphisms with characteristics similar to PM182. We refer to these as restriction site (RS) polymorphisms. Again, we failed to see three-banded individuals at these polymorphisms suggesting that the variable restriction sites are located outside the coding region that hybridizes with the probe. Because each probe used in the study was used to score only one single-locus polymorphism, the designation of the probe is also used to designate the polymorphic locus detected by that probe. A more detailed description of these polymorphisms appears in Pogson (1994).

We have used three approaches in testing the null hypothesis that there is no class-related effect on the difference in shell height among individuals with differing degrees of heterozygosity. First, all 17 loci were treated as comprising one homogeneous class and shell height was regressed against the degree of individual heterozygosity. Then the loci were divided into two mutually exclusive groups and the two regressions were compared to each other and to the overall regression.
Figure 2.—Shell heights of 222 scallops plotted against the degree of heterozygosity measured over the 17 loci listed in Table 1. Bold circles indicate the mean shell height of each heterozygosity class. The solid line is the regression of individual shell height against the degree of heterozygosity.

This is the usual type of analysis in studies relating the degree of heterozygosity with phenotypic traits and is performed here for the purpose of providing a common basis for comparison of our results with previous studies on marine bivalves. The second method is the Wilcoxon two-sample non-parametric test. For this test, the relative contribution of an individual locus to the regression between shell height and the degree of heterozygosity (measured over all seventeen loci) was evaluated from the partial sums-of-squares resulting when homozygosity/heterozygosity for that locus was entered as an independent variable (cf. Kohne et al. 1988). We used the Wilcoxon test to compare the rank ordering of different classifications of loci (see Table 1 for listing of ranks). In all applications the Kruskal-Wallis (1952) correction for continuity was applied. The third test is the Wilcoxon test to compare the rank ordering of different combinations of loci (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Classification</th>
<th>n</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All loci</td>
<td>17</td>
<td>0.071</td>
<td>0.293</td>
</tr>
<tr>
<td>Enzyme loci</td>
<td>7</td>
<td>0.148</td>
<td>0.028</td>
</tr>
<tr>
<td>Protein loci</td>
<td>9</td>
<td>0.119</td>
<td>0.077</td>
</tr>
<tr>
<td>Non-enzyme loci</td>
<td>10</td>
<td>-0.033</td>
<td>0.626</td>
</tr>
<tr>
<td>Non-protein loci</td>
<td>8</td>
<td>-0.019</td>
<td>0.778</td>
</tr>
<tr>
<td>Point mutation loci</td>
<td>12</td>
<td>0.109</td>
<td>0.105</td>
</tr>
<tr>
<td>VNTR loci</td>
<td>5</td>
<td>-0.048</td>
<td>0.480</td>
</tr>
<tr>
<td>Point mutation, non-enzyme loci</td>
<td>5</td>
<td>0.001</td>
<td>0.996</td>
</tr>
</tbody>
</table>

Regressions are of shell height against the degree of heterozygosity measured over all loci, or over subsets of loci, in the sample of 222 individuals. n = number of loci; r = correlation coefficient; P = probability that r is different from zero (from the F statistic with degrees of freedom of 1 and 220).

Figure 3 plots the shell heights of all 244 individuals against their degree of allozyme heterozygosity. The correlation between these variables is significant despite accounting for less than 3% of observed variance in growth rate (r = 0.163, P = 0.011). The results comparing the two sets of loci are shown in Table 3. Given the prior knowledge that the enzyme loci produce a positive correlation with growth rate, the probabilities given in the table are from one-tailed tests. The two parametric correlation coefficients are significantly different at the 5% level. The Wilcoxon non-parametric test on the ranks of the relative contributions of individual loci to the regression produced a similar result (Table 3).

An inspection of the effects of individual loci on the heterozygosity-growth correlation (Table 1) reveals that a few enzyme loci rank low (e.g., Pmi) and a few RFLP loci rank high (e.g., PM238), whereas for some other pairs the difference is very small (e.g., Pgd and PM182). In the latter case the two loci still receive a different rank number, and this affects the result of the Wilcoxon test. To avoid this limitation of non-parametric tests, we returned to the correlation between heterozygosity and shell height and asked how many of all possible combinations of seven loci out of the seventeen produce a correlation that equals or exceeds the one produced by the seven allozyme loci. Of all 19,448 possible combinations, 1,061 did (5.5%, Table 3). This suggests that there is about a 5% chance that any random set of genetic markers could generate a correlation as good as the one seen in allozyme studies. These 1,061 combinations are, however, highly biased in regard to the number of allozyme loci (of the seven) they contain. This is shown in Figure 4 which lists the number of combinations with 0–7 allozyme loci expected among all
whose correlation coefficient exceeded that of the loci. Rest of notation as in Figure 3. It can be seen that the observed to expected ratio numbers actually observed in the specific collection of such combinations in a random sample of 1,061, and the expected number of combinations with two or fewer enzyme loci in a random collection of 1,061 is 375.46, but only 18 of these were present among the 1,061 combinations whose correlation coefficient exceeded that of the 7 allozyme loci.

possible 19,448 combinations, the expected number of such combinations in a random sample of 1,061, and the numbers actually observed in the specific collection of 1,061 with the highest heterozygosity-growth correlations. It can be seen that the observed to expected ratio decreases rapidly with the number of enzyme loci in the combination. For example, the expected number of combinations with two or fewer enzyme loci in a random collection of 1,061 is 375.46, but only 18 of these were present among the 1,061 combinations whose correlation coefficient exceeded that of the 7 allozyme loci.

Possible 19,448 combinations, the expected number of such combinations in a random sample of 1,061, and the numbers actually observed in the specific collection of 1,061 with the highest heterozygosity-growth correlations. It can be seen that the observed to expected ratio decreases rapidly with the number of enzyme loci in the combination. For example, the expected number of combinations with two or fewer enzyme loci in a random collection of 1,061 is 375.46, but only 18 of these were present among the 1,061 combinations whose correlation coefficient exceeded that of the 7 allozyme loci.

Coding vs. non-coding loci: The assumption of neutrality for the RFLP loci may not apply to loci that code for nonspecific proteins. In this respect, the two nonspecific loci may be more closely aligned with the enzyme than the RFLP loci. All 244 individuals scored for the seven allozymes were also scored for these two loci. The regression of shell height against the degree of heterozygosity at this new set of nine loci is significant at the 5% level. On the sample of 222 individuals, the probability is nearly 8%. The complementary set of eight nonspecific proteins (plotted in Figure 5) produces a correlation coefficient of zero, and the probability that this coefficient is different from that for the nine protein-coding loci is 0.075 (Table 3). Unfortunately, the small number of nonspecific protein loci prevents a meaningful comparison with the seven allozyme loci. Neither the parametric comparison, nor the Wilcoxon test were significant (Table 3), even though six of the seven enzymes outscored both nonspecific protein loci (Table 1).

Point mutation vs. VNTR loci: Even if the polymorphisms that we have surveyed are all selectively neutral, the probability that they will be in transient linkage disequilibrium with deleterious genes can still be different since the mutational mechanisms generating these polymorphisms differ. The decay of disequilibrium is expected to be faster when the mutation rate is high and when the probability of producing the same allele by independent mutational events is also high. In this respect, the VNTR loci form a special category, as they are suspected to arise by entirely different mechanisms than the allozymes or restriction site polymorphisms [see JEFFREYS et al. (1988) and JARMAN and WELLS (1989)] which are both produced by point mutation. The statistics treating the VNTR loci as one group (Figure 6) and the other loci as another are summarized in Tables 2 and 3. There is no evidence for a correlation between the de-

![Figure 3](image-url)

**Figure 3.** Shell heights of 244 scallops plotted against the degree of heterozygosity measured over the seven enzyme loci. Open circles correspond to 22 animals not scored for the DNA polymorphisms. Rest of notation as in Figure 2.

![Figure 4](image-url)

**Figure 4.** Shell heights of 222 scallops plotted against the degree of heterozygosity measured over the 10 non-enzyme loci. Rest of notation as in Figure 2.

### Table 3

<table>
<thead>
<tr>
<th>Comparison</th>
<th>( r_1/r_2 )</th>
<th>Wilcoxon</th>
<th>RCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme (7)/non-enzyme (10)</td>
<td>0.028</td>
<td>0.049</td>
<td>0.055</td>
</tr>
<tr>
<td>Protein (9)/non-protein (8)</td>
<td>0.075</td>
<td>0.283</td>
<td>0.125</td>
</tr>
<tr>
<td>Point mutation (12)/VNTR (5)</td>
<td>0.050</td>
<td>0.232</td>
<td>0.149</td>
</tr>
<tr>
<td>Enzyme (7)/VNTR (5)</td>
<td>0.020</td>
<td>0.052</td>
<td>0.081</td>
</tr>
<tr>
<td>Non-enzyme point mutation (5)/VNTR (5)</td>
<td>0.308</td>
<td>0.266</td>
<td>0.349</td>
</tr>
<tr>
<td>Enzyme (7)/non-enzyme point mutation (5)</td>
<td>0.062</td>
<td>0.146</td>
<td>0.112</td>
</tr>
<tr>
<td>Enzyme (7)/nonspecific proteins (2)</td>
<td>0.402</td>
<td>0.094</td>
<td>0.139</td>
</tr>
</tbody>
</table>

The first column \( (r_1/r_2) \) lists the probability that the two parametric correlation coefficients (from Table 2) are significantly different. Probabilities listed in the second column are from the Wilcoxon test and in the third from the RCT. All are one-tailed probabilities, testing the null hypothesis that the correlation of shell height with the degree of heterozygosity at the second class of loci is not different from that of the first (see text for details). Numbers in parentheses are the numbers of loci in each class.
Allozyme and RFLP Heterozygosity

TABLE 4

Results from the random combination test

<table>
<thead>
<tr>
<th>No. of enzyme loci in the combination</th>
<th>Total among all possible combinations</th>
<th>Expected in a random set of 1,061</th>
<th>Observed among the 1,061 with the highest correlation</th>
<th>Ratio of observed to expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>120</td>
<td>6.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1,470</td>
<td>80.20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5,292</td>
<td>288.71</td>
<td>18</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>7,350</td>
<td>400.98</td>
<td>359</td>
<td>0.90</td>
</tr>
<tr>
<td>4</td>
<td>4,200</td>
<td>229.13</td>
<td>492</td>
<td>2.15</td>
</tr>
<tr>
<td>5</td>
<td>945</td>
<td>51.55</td>
<td>177</td>
<td>3.43</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>3.62</td>
<td>15</td>
<td>3.93</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>19,448</td>
<td>1,061</td>
<td>1,061</td>
<td></td>
</tr>
</tbody>
</table>

All possible combinations of 7 loci drawn from the pool of 17 are classified according to the number of enzyme loci they contain. The expected number of each class in a random subset of 1,061 combinations is compared to the observed number in the specific subset of combinations that produced a correlation better than that produced by the unique subset of seven enzymes.

Figure 5.—Shell heights of 222 scallops plotted against the degree of heterozygosity measured over the eight RFLP loci. Rest of notation as in Figure 2.

Figure 6.—Shell heights of 222 scallops plotted against the degree of heterozygosity measured over the five VNTR loci. Rest of notation as in Figure 2.

Heterozygosity with shell height for either group or that one group produces a stronger correlation than the other.

Heterozygote deficiencies: In addition to providing information on heterozygosity and shell height, Table 1 also details the amounts of polymorphism and deviations from Hardy-Weinberg expectations. A wide range of variability is covered by the 17 loci sampled. Observed heterozygosity ranged from 4 to 93% and the number of alleles observed per locus ranged from 2 to 41. At 13 loci there was a deficiency in the number of heterozygotes, but the overall deficiency (equivalent to Wright's $F_{is}$, with changed sign) was only $-0.037$. This level of heterozygote deficiency is significantly different from zero ($P = 0.020$), but is smaller than generally observed in other marine bivalve species (Zouros and Foltz 1984; Gaffney 1990). The magnitude of heterozygote deficiency observed is, however, in agreement with previous studies on this species. Six of the allozymes examined in the present study (Pgm, Pgi, Odh, Pmi, Pgd and Got) were also surveyed by Volckaert and Zouros (1989) in six cohorts of juveniles sampled from the same population between November 1984 and December 1985. The mean heterozygote deficiency at these loci over all samples ($-0.065$) was similar to that seen for the same loci in our study ($-0.044$). Locus-specific deficiencies are strongly correlated with the exception of Got which did not show a deficiency of heterozygotes in the present study. These observations suggest that no significant changes have occurred in the genetic structure of the population between the two studies. There is no heterogeneity between the deficiencies observed among enzyme and non-enzyme loci ($\chi^2 = 12.0$, with 16 d.f., $P = 0.744$). In contrast to the Mulinia study by Gaffney et al. (1990) in which a comparable number of loci were scored, no correlation was observed between the mag-
nitude of heterozygote deficiency at a particular locus and its effect on the heterozygosity-growth correlation. This holds for the entire set of loci ($r = -0.008, P = 0.977$) as well as for the seven allozyme loci ($r = -0.038, P = 0.936$).

DISCUSSION

This study has attempted to test the hypothesis that in studies relating the degree of heterozygosity with fitness characters the enzyme loci scored act as neutral markers of linked deleterious genes. According to this explanation (the associative overdominance hypothesis), a second independent set of marker loci, whose selective neutrality is not seriously in doubt, is expected to produce a similar correlation between heterozygosity and the fitness character, here measured as shell height in a cohort of the deep-sea scallop, *P. magellanicus*. When the old and new sets of loci are combined one would expect the new index of individual heterozygosity to produce a stronger correlation with shell height than each set of markers separately. Failure to see a correlation with the new set of markers would be incompatible with the above hypothesis. Our study has shown that in contrast to seven allozyme loci, no correlation was observed between growth rate and the degree of heterozygosity scored at the set of 10 new loci or over the combined set of 17 loci. This result provides evidence against the hypothesis that the allozyme loci are acting as neutral markers of linked deleterious genes.

This conclusion is based on the assumption that the ten new non-enzyme loci are as likely to be involved in non-random genotypic associations with loci segregating for deleterious genes as the seven allozyme loci. Genotypic correlation at two or more loci may have two causes: gametic phase disequilibria or non-random union of gametes at the formation of zygotes (one-generation inbreeding effect). The latter process will not discriminate between enzyme and non-enzyme loci, so one is justified in assuming that the contributions of the two classes of loci to the heterozygosity-growth correlation through genotypic associations generated by in-breeding will be similar. Gametic phase disequilibria may, however, vary among marker loci. For example, a DNA polymorphism situated in a large non-functional segment of the genome (such as a heterochromatic region) will have a much lower probability of being in the proximity of a locus affecting growth than an enzyme or non-specific protein locus. Our cDNA probes were chosen to minimize this possibility. The high specificity of each allelic set of restriction fragments to the corresponding cDNA probe ensures that the RFLPs scored, whether due to the gain or loss of restriction sites or to varying numbers of repeated sequences, are closely linked to transcribed regions. An upper estimate of the distance between the transcribed site and the location of the polymorphism is given by the size of the DNA fragments comprising the polymorphism, which in our study did not exceed 15 kb. Given that one recombination unit (1 cM) is estimated to correspond to two megabases, our RFLPs are as likely to be involved in recombination linkages with an unscored locus affecting growth rate as are the enzyme loci. The same is true for the two nonspecific proteins.

Two neutral marker loci separated from a selected locus by the same recombination distance may still have different probabilities of being in linkage disequilibrium with the selected locus if the underlying mutational mechanisms operating at the markers are different. As a decoupling force, mutation will be less potent if its rate is low and if each mutational event generates a new allele in the population. Mutation rates at VNTR loci can be very high, as large as $5 \times 10^{-2}$ (Jeffreys et al. 1988), although we cannot know if this estimate applies to our VNTR loci. This rate must be compared to $10^{-6}$ to $10^{-7}$ for allozyme loci (e.g., Mukai and Cockerm 1977; Voelker et al. 1980). The mutation rate of restriction site polymorphisms is most likely comparable to that for allozymes (assuming that the DNA fragments detected by our probes are as large as 15 kb, that the mutation rate per nucleotide is as high as $5 \times 10^{-9}$, and that one in a thousand point mutations results in the gain or loss of a restriction site detectable by a four-cutter gives a mutation rate of $10^{-7}$). More importantly, while the infinite sites or alleles model can be considered as a satisfactory approximation for the point mutation polymorphisms, it probably cannot for the VNTR polymorphisms. All proposed mechanisms for the generation of new VNTR alleles (Jarman and Wells 1988) are likely to produce repeat arrays with copy numbers similar to ones already existing in the population. Indirect evidence that this might be true for our VNTR loci comes from the observation that their allele frequency distributions are unimodal, with intermediate-sized alleles being most frequent.

Based on these considerations, the comparison of point mutations to VNTR polymorphisms is of special interest. As shown in Table 3, the correlation coefficients of shell height with the degree of heterozygosity at the two classes of loci are different at the conventional level of significance, but neither the Wilcoxon nor the random combination test produces a significant result. Therefore, there is not sufficient evidence for the possibility that mutational mechanism affects the heterozygosity-fitness correlation. The five VNTR loci did, however, distinct from the seven allozyme loci but indistinguishable from the five non-enzyme point mutation loci. This is compatible with the view that the difference between the VNTR and point mutation polymorphisms is not due to mutational mechanism but to the stronger influence of the enzyme loci on the heterozygosity-growth correlation. The most informative comparison in this respect is that between the allozyme loci and the five non-enzyme point mutation
loci, which borders on significance (Table 3). Our results provide no evidence for a difference between the point mutation and VNTR loci but indicate that the allozymes are the only subset with a consistently higher effect on the heterozygosity-growth correlation. Clearly, our evidence against the associative overdominance hypothesis depends critically on the inclusion of the VNTR loci in the list of valid markers in linkage disequilibria with presumed deleterious genes, but neither the statistical power of our tests nor our present state of knowledge about the origin and maintenance of VNTR polymorphisms are sufficient to decide the issue.

It can be seen from Table 1 that the mean shell heights of heterozygotes exceeded homozygotes at 8 out of the 17 loci and that 5 of these 8 are allozymes. With the notable exception of Pmi, the enzyme loci appear indeed to be more homogeneous than any other group, not only with regard to sign but also with regard to the size of this difference. Negative contributions to the heterozygosity-growth correlation of a few enzyme loci have been noticed in previous studies on marine bivalves (Gaffney 1990). Koehn et al. (1988) suggested that the variation in the effects of individual loci, although compatible with the associative overdominance hypothesis, in fact provides evidence favoring the direct involvement of allozymes to the heterozygosity-growth correlation. They observed that among the fifteen enzyme loci surveyed, those involved in glycolysis (or pathways feeding into glycolysis) and protein catabolism (type I enzymes) had a stronger effect on the correlation than loci functioning in alternative pathways (type II enzymes), and reasoned that this is explicable on the basis of previous observations that energy expenditure and protein turnover of basal metabolism is apparently lower in individuals with high degrees of heterozygosity (Koehn and Shumway 1982; Hawkins et al. 1986). This classification of enzyme loci does not, unfortunately, explain the strong negative effect of Pmi in our study, which is a preglycolytic enzyme. Viewed as a whole, our limited set of seven enzyme loci provides neither positive nor negative evidence for the distinction between type I and type II enzymes.

Restriction site polymorphism PM238 also requires comment. At this locus the difference between the mean shell height of heterozygotes and homozygotes is the largest of the 17 loci and (together with Adh) statistically different from zero. Since this is a restriction site polymorphism, it is possible that the variable site falls within a coding region and is in non-random association with functional alleles that are themselves responsible for the difference in shell height. However, population studies of DNA sequence variation at enzyme loci in Drosophila have produced contradictory evidence for intragenic linkage disequilibria among synonymous substitutions, or among synonymous and non-synonymous substitutions (Aquadro et al. 1986; Simmons et al. 1989; Riley et al. 1989, 1992), so it is difficult to favor this explanation over others (such as linkage with a non-allelic gene or that the difference is simply a chance event).

Two other studies have attempted to correlate growth rate and the degree of enzyme heterozygosity in the deep-sea scallop, P. magellanicus. Foltz and Zouros (1984) examined two samples from an adult population. Although they found a correlation of heterozygosity with age, they failed to observe a significant relationship within individual age classes. More relevant to the present study is that of Volckaert and Zouros (1989) who examined six samples of juvenile scallops from the same natural population as the one examined here. In all six of their samples the correlation between growth rate and the degree of heterozygosity at six enzyme loci was positive, but in only one was it statistically significant. The overall correlation coefficient was 0.058 (on n = 1138) which is not different from the one obtained here on seven allozyme loci (P = 0.22).

It is useful to compare the results of the present study with that of Koehn et al. (1988) and Gaffney et al. (1990) on the cot clam, M. laevis. The two studies have employed twice as many loci (17 and 15, respectively) than is usually the case, but have produced quite different results. In the Mulinia study, the mean shell height of heterozygotes was larger than homozygotes at 15 of the 17 loci, and in 8 of these cases the difference was significant. In the present study, the difference was positive at 8 loci and significant at only 2. In the Mulinia study there was a deficiency of heterozygotes at all but one locus and in only one case was the deficiency not significant. In our study, heterozygote deficiencies occurred at 13 loci but only 1 (Pgm) was significant. Furthermore, in the Mulinia study there was a strong relationship between the effect of a locus on the correlation and the magnitude of heterozygote deficiency at that locus, but no such relationship was apparent in the scallop data. Although the Mulinia study employed a sample size that was nearly 10 times larger than the present study, it is unlikely that these differences would disappear if our sample had been much larger. It is more tempting to ascribe these differences to the loci used in the two studies. The Mulinia study used allozyme loci exclusively and produced uniform and predictable results. Our study incorporated both enzyme and DNA markers and produced heterogeneous and unpredictable results.

In conclusion, our results indicate that growth rate in juvenile scallops is related to the degree of allozyme heterozygosity, a result that is well documented in other marine bivalves. We have attempted to extend this result to another index of individual heterozygosity without success. The allozymes were the only group of loci that produced a significant and consistent correlation. Since 8 of the 10 non-enzyme loci can be safely considered to be neutral to the forces of selection acting on the individual, this behavior of the enzyme loci suggests that they
are not merely acting as neutral markers in studies relating heterozygosity and fitness characters. Several qualifications need to be made regarding this conclusion. First, VNTR loci may not provide a valid set of loci to test the associative overdominance hypothesis of linkages with deleterious genes. Second, our inclusion of only two nonspecific protein loci does not allow a firm distinction to be made between loci coding for enzymes and loci coding for non-enzyme proteins. Unfortunately, it appears that in general only a small fraction of nonspecific proteins exhibit polymorphism that can be detected by standard electrophoresis (Singh and Coulthart 1982). Third, studies of correlations between heterozygosity and phenotypic characters are known to have a low degree of reproducibility. This can be interpreted to mean that the correlation does not exist as a biological phenomenon. It might also be the pattern we expect to see, given that 6-12 enzymes usually employed in such studies must represent a small fraction of all loci that affect the correlation (Mitton and Pierce 1980; Chakraborty 1981). It might not be accidental that even the most successful demonstrations of the correlation (Zouros et al. 1980; Koehn and Gaffney 1984; Koehn et al. 1988) fail to explain more than 5% of the phenotypic variance. For these reasons, a conclusion as important as the one claimed in this report cannot be generalized before similar studies are performed in other species.

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LITERATURE CITED


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