

## Ecological Genetics of Mpi and Gpi Polymorphisms in the Acorn Barnacle and the Spatial Scale of Neutral and Non-neutral Variation<sup>1</sup>

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**SYNOPSIS.** Different allozyme genotypes at the mannose phosphate isomerase (Mpi) locus in the northern acorn barnacle (*Semibalanus balanoides*) show a strong association with distinct intertidal microhabitats. In estuaries along the Maine Coast, the FF homozygote has higher fitness in exposed, high-tide level microhabitats while the SS homozygote has higher fitness under algal cover or at low-tide microhabitats. These patterns are consistent with a Levene (1953) model of balancing selection. In these same samples, polymorphisms at the glucose phosphate isomerase locus (Gpi) and mitochondrial DNA (mtDNA) show no fitness differences among microhabitats, providing intra-genomic controls supporting selection at or near Mpi. Here we report a similar analysis of genotype-by-microhabitat associations at sites in Narragansett Bay, Rhode Island, close to the southern range limit of *S. balanoides*. Genotype zonation at Mpi between high- and low-tide microhabitats is significantly different between Maine and Narragansett Bay due to opposite zonation patterns for the SF and FF genotypes. Enzyme activity data are consistent with this “reverse” zonation. At Gpi, there is significant microhabitat zonation in Narragansett Bay, while this locus behaves as a neutral marker in Maine. Mt DNA shows no significant microhabitat zonation in either Rhode Island or Maine. The Mpi data suggest that Levene-type selection for alternative genotypes in alternative habitats may operate at scales of both 10’s of meters and 100’s of kilometers. The Gpi data show how an apparently neutral locus can exhibit non-neutral variation under different environmental conditions. We argue that both Mpi and Gpi provide important genetic variation for adaptation to environmental heterogeneity that is recruited under distinct conditions of stress and carbohydrate substrate availability.

### INTRODUCTION

The role of genetic variation in adaptation to environmental heterogeneity has been a controversial topic in evolutionary biology for decades. The enzyme polymorphisms of central metabolism have been prime suspects in this debate (*e.g.*, compare Lewontin, [1991] and Watt [1994]). Since the observed variation on “allozyme” gels is due to charge differences in functional proteins, simple intuition leads to the suspicion that allozyme alleles have some association with fitness. When these presumed functional differences are distributed throughout natural microhabitats “. . . it seems only common sense that a population should be more diverse genetically if it lives in a variable environment.” (Maynard Smith, 1998, p. 70). Despite considerable effort to uncover the adaptive potential of allozyme variation, the null hypothesis that many of these polymorphisms are simply neutral variants on a trajectory to fixation or loss (Kimura and Ohta, 1971) has survived remarkably well in the face of the data.

While this generalization may hold for the broad class of allozyme variation, there are a number of specific loci where the evidence against neutrality is compelling. For example, the *Lap* locus in the blue mussel (Koehn and Hilbish, 1987); *Gpi* locus in *Colias* butterflies (Watt, 1977; Watt *et al.*, 1985); *Adh* in *Drosophila* (Oakshott *et al.*, 1982; Kreitman and Hudson,

1991); *Ldh* in *Fundulus* (Crawford and Powers, 1989) all show patterns of variation or genotype specific functional differences that suggest selection (see many other examples in Mitton, 1997; Hochachka and Somero, 2002). But the issue of genetic variation in heterogeneous environments goes beyond evidence for selection: one must show that the net effect of selection can actively maintain the polymorphism.

Selection is inferred when replicate populations show similar patterns of genotype-by-habitat association (*e.g.*, the *Lap* polymorphism shows clinal variation across salinity gradients in multiple estuaries Koehn *et al.*, 1976). The case for selection is strengthened by comparing patterns of variation among loci. Drift and migration act on all loci in the genome, but selection acts in a locus-specific manner (Cavalli-Sforza, 1966; Lewontin and Krakauer, 1973). When all of the loci in question are allozymes, this approach can suffer from an inability to objectively determine which loci should, and should not, respond to selection. However, when inter-locus contrasts are made between polymorphisms with potential functional significance (*e.g.*, allozymes) and reference loci that can be determined, *a priori*, to be effectively neutral (*e.g.*, non-coding intergenic regions), the evidence for selection can be unassailable (*e.g.*, Berry and Kreitman, 1993).

The selective maintenance of polymorphism is more restrictive. The common models of balancing selection involve heterosis, marginal overdominance from a multiple-niche polymorphism (Levene, 1953), frequency-dependent selection, and habitat selection, among others (Powell and Taylor, 1979; Hedrick, 1986). A crucial issue in the first two of these mech-

<sup>1</sup> From the Symposium *Physiological Ecology of Rocky Intertidal Organisms: From Molecules to Ecosystems* presented at the Annual Meeting of the Society for Comparative and Integrative Biology, 2–7 January 2002, in Anaheim, California.

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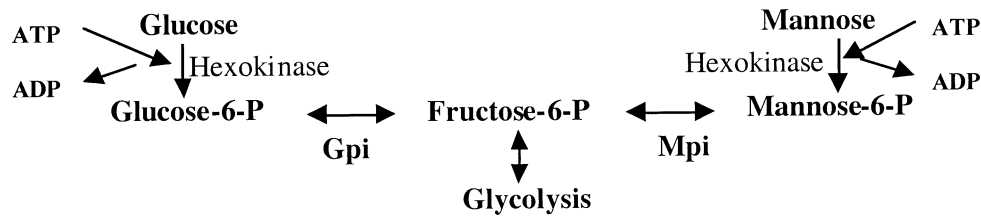


FIG. 1. Simplified pathways of hexose metabolism as related to Mpi and Gpi. Note that Mpi and Gpi share a product in the forward direction and a substrate in the reverse direction (fructose-6-phosphate).

anisms is the spatial scale of fitness variation. In simple heterosis, the environment is heterogeneous on a small scale, all genotypes experience all microenvironments, and it so happens that heterozygotes have the highest fitness. In the Levene model (Levene, 1953) of marginal overdominance, the environment is heterogeneous on a coarse scale, and alternative genotypes have different fitnesses in different niches. Polymorphism can be maintained with the appropriate fitnesses and proportions of the various niches. There is a sizeable literature describing the correlation between multi locus heterozygosity and growth rate in bivalves (*e.g.*, Pogson and Zouros, 1994 and references therein), but this relationship may not extend to other organisms (*e.g.*, Houle, 1989). Direct experimental evidence for Levene (1953) type selection in natural populations is quite rare.

Recent studies of the northern acorn barnacle (*Semibalanus balanoides*) have provided evidence for balancing selection at the Mpi allozyme locus using a number of the approaches outlined above. There is strong Mpi genotype zonation between thermally stressed and non-stressed intertidal microhabitats across multiple sites and years in Maine estuaries (Schmidt and Rand, 1999). This zonation is not observed at the Gpi locus or a neutral mtDNA RFLP, indicating selection at Mpi or a linked locus. Experimental transplants of newly settled barnacles show that the Mpi-FF genotype survives better in thermally stressed microhabitats, but that the Mpi-SS genotype survives better at the thermally benign extreme of the intertidal zone (Schmidt *et al.*, 2000). Both the Gpi and mtDNA markers show no fitness effects in this experiment. By applying these genotype-specific fitnesses for Mpi to the Levene (1953) model, conditions for the stable maintenance of the polymorphism are met (Schmidt *et al.*, 2000). Moreover, Mpi, Gpi and mtDNA show no microhabitat zonation at larval settlement in early April. Gpi and mtDNA frequencies do not change as the cohort ages, but Mpi exhibits increasing degrees of genotype zonation through May and June (Schmidt and Rand, 2001). This rules out habitat selection as an explanation for genotype zonation. The lingering issue of selection at a linked locus is addressed by experiments in which alternative Mpi genotypes show significantly different growth rates when supplemented with mannose, but not with fructose or a no-sugar control (Schmidt, 2001). Since there is no evidence for Mpi isozymes in *Semibalanus*

(Flowerdew, 1983), and a random linked locus should not affect growth in a substrate-dependent manner, selection at the Mpi locus itself is the most parsimonious explanation for the data (Schmidt, 2001).

A striking aspect of allozyme variation in *S. balanoides* is that Mpi and Gpi are the only two enzymes of central metabolism that show a strong two-allele polymorphism (Flowerdew, 1983). Because both Mpi and Gpi are important parallel feeder pathways in carbohydrate metabolism (see Fig. 1), genetic variation at these loci may provide biochemical flexibility for adaptation to environmental heterogeneity. The relative importance of these loci will depend on the thermal stability of the allozymes, the availability of alternative sugars in the diet, and the contribution of each locus (and genotypes at each locus) to fitness in terms flux through glycolysis (*e.g.*, Watt, 1994). Since substrate availability does have important fitness consequences at Mpi (Schmidt, 2001), geographic variation in substrate availability may be an important component in the fitness variation governing polymorphisms at both Mpi and Gpi. Evidence for geographic variation in genotype-by-microhabitat associations is a prerequisite for these mechanistic hypotheses, which is the focus of the current study.

In this paper we examine the ecological genetics of *S. balanoides* on a biogeographic scale through statistical comparisons of Mpi, Gpi and mtDNA variation between Maine and Rhode Island. Studies of *S. balanoides* in the Gulf of St. Lawrence show that both Mpi and Gpi exhibit significant allele frequency shifts suggestive of selection (Holm and Bourget, 1994; Dufresne *et al.*, 2002). Because Rhode Island lies near the southern limit of *S. balanoides* (Wetthey, 1983) the thermal stress should be more extreme (*e.g.*, Bertness and Gaines, 1993). Since our earlier studies in Maine show that Gpi behaves as a neutral locus, tests for Mpi and Gpi zonation in Rhode Island will help resolve the biogeographic scale of neutral and non-neutral variation at these important enzymes. If temperature stress is a key factor governing genetic variation at Mpi and Gpi, the higher average temperature in Rhode Island leads to some simple predictions. For Mpi, the frequency of the Fast allele should be higher, and the intertidal zonation of genotypes should be more pronounced than in Maine. For Gpi, the conflicting results between Canada and Maine may be reconciled if a cline in frequency can be extended south to Rhode Island. The data reported here do not support this sim-

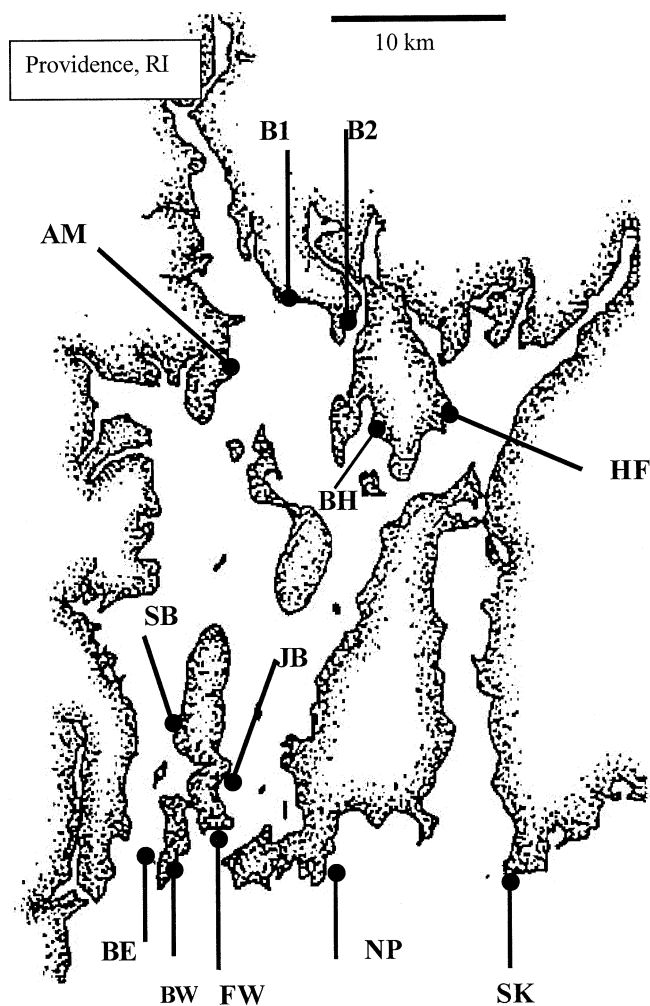


FIG. 2. Map of collecting localities in Narragansett Bay, Rhode Island. See Materials and Methods and Table 1 for definitions of abbreviations.

ple temperature hypothesis but reveal that genotype zonation is opposite in direction for Southern vs. Northern New England. The lack of zonation at the neutral mtDNA marker in both regions indicates that selection for alternative allozyme genotypes in alternative habitats is operating at both the microhabitat and biogeographic scale.

#### MATERIALS AND METHODS

##### Sampling

Collecting localities in Narragansett Bay, Rhode Island are shown in Figure 2 and listed in Table 1. Data from Maine are from Table 1 of Schmidt and Rand (1999). In May and June of 2000, adult barnacles were collected at four localities in Narragansett Bay: Aldrich Mansion (AM), Bristol Harbor (BH), Jamestown Beach (JB), and Seaside Beach (SB). These localities are referred to as "Bay" sites; see Figure 2. Two additional localities were sampled in 2000 at exposed sites on the coast: Sakonnet (SK) and Fort Wetherill (FW). These are referred to as "Coast" sites. In 1993

adult barnacles were collected from three additional Bay localities (Barrington 1, B1; Barrington 2, B2; Haffenreffer, HF), and three coast localities (Beavertail East, BE; Beavertail West, BW; Newport, NP). At each locality barnacles were collected from two microhabitats chosen to represent extremes of desiccation and heat stress. The "High" microhabitat was in the upper 6 inches of the high intertidal zone on boulders with west- or south-facing aspects giving maximal exposure to afternoon sun. The "Low" microhabitat was in the lowest intertidal zone on rocky substrate or cobbles with a north-facing aspect. Within each locality, the High and Low microhabitats were separated by 2–10 m. Barnacles were collected along a transect of approximately 20 horizontal feet within each tidal height. Previous studies at other Narragansett Bay localities indicate that thermal stress is much greater at high-tide locations (Bertness and Gaines, 1993; see also Wethey, 2002 in this issue). The 1993 localities Haffenreffer, Newport and Beavertail East differ slightly in having east-facing aspects, but barnacles were still collected at tidal height extremes. All barnacles collected were individuals from the previous year's larval settlement, and possibly some individuals from two previous years. Thus, all barnacles sampled had experienced at least one year of exposure of post-settlement heat and desiccation stress during the summer months and any possible fitness costs of reproduction, which occurs through the fall. Barnacles were kept on ice until storage at  $-80^{\circ}\text{C}$  in the lab.

##### Genotype analysis

All allozyme and mtDNA analyses were done as described in Schmidt and Rand (1999). Barnacle tissue was homogenized in deionized  $\text{H}_2\text{O}$  and centrifuged at  $\sim 3,000$  rpm in a microfuge to pellet cellular debris. Five  $\mu\text{l}$  of supernatant was added to the wells of an ice-cold 12-sample well plate, and then applied to cellulose acetate electrophoresis plates using a Helena Laboratories Super Z-12 applicator. Proteins were electrophoresed for 15 min at 100 volts and stained with appropriate substrates and cofactors as described in Hebert and Beaton (1989). The mtDNA analyses also followed procedures of Schmidt and Rand (1999). Briefly, 50  $\mu\text{l}$  of homogenate were added to 50  $\mu\text{l}$  of 10 mM Tris (pH 8.2), 1 mM EDTA, and 25 mM NaCl buffer with Proteinase K added to a final concentration of 2  $\mu\text{g}/\mu\text{l}$ . This mixture was incubated for one hour at  $37^{\circ}\text{C}$  and then proteins were denatured at  $95^{\circ}\text{C}$  for 3 min. PCR amplification of the *S. balanoides* D-loop used the primers and cycle parameters described in Schmidt and Rand (1999). A polymorphic *Dde* I restriction site lies in the *S. balanoides* D-loop defining two haplotypes, A and B (see Schmidt and Rand [1999] and Brown *et al.* [2001]).

##### Statistical analysis

Allelic and genotypic variation was determined across multiple scales. First, we compared variation between High- and Low-tide microhabitats at each site

TABLE 1. Genotype and allele frequencies for *Mpi* and *Gpi* in Narragansett Bay, Rhode Island.

Date	Region	Locality	Site	Mpi					Gpi						
				n	f(SS)	f(SF)	f(FF)	f(S)	f(F)	n	f(SS)	f(SF)	f(FF)	f(S)	f(F)
2000	Bay	Bristol Harbor	High	82	0.13	0.56	0.30	0.415	0.585	88	0.51	0.31	0.18	0.665	0.335
2000	Bay	Bristol Harbor	Low	79	0.20	0.38	0.42	0.392	0.608	84	0.40	0.48	0.12	0.643	0.357
2000	Bay	Aldrich Mansion	High	53	0.15	0.36	0.49	0.330	0.670	55	0.45	0.44	0.11	0.673	0.327
2000	Bay	Aldrich Mansion	Low	29	0.10	0.45	0.45	0.328	0.672	30	0.33	0.57	0.10	0.617	0.383
2000	Bay	Jamestown Beach	High	66	0.15	0.52	0.33	0.409	0.591	71	0.49	0.39	0.11	0.690	0.310
2000	Bay	Jamestown Beach	Low	61	0.10	0.43	0.48	0.311	0.689	64	0.38	0.52	0.11	0.633	0.367
2000	Bay	Seaside Beach	High	69	0.10	0.49	0.41	0.348	0.652	70	0.54	0.37	0.09	0.729	0.271
2000	Bay	Seaside Beach	Low	84	0.13	0.39	0.48	0.327	0.673	94	0.44	0.47	0.10	0.670	0.330
2000	Bay	Total	High	270	0.13	0.49	0.37	0.380	0.620	284	0.50	0.37	0.13	0.688	0.312
			Low	253	0.14	0.40	0.45	0.344	0.656	272	0.40	0.49	0.11	0.647	0.353
			Pooled	523	0.14	0.45	0.41	0.362	0.638	556	0.45	0.43	0.12	0.668	0.332
2000	Coast	Sakonnet	High	78	0.10	0.49	0.41	0.346	0.654	91	0.40	0.51	0.10	0.648	0.352
2000	Coast	Sakonnet	Low	54	0.09	0.41	0.50	0.296	0.704	54	0.46	0.33	0.20	0.630	0.370
2000	Coast	Fort Wetherill	Stressed	45	0.07	0.42	0.51	0.278	0.722	40	0.55	0.35	0.10	0.725	0.275
2000	Coast	Fort Wetherill	Low	59	0.10	0.42	0.47	0.314	0.686	60	0.37	0.42	0.22	0.575	0.425
2000	Coast	Beavertail	Low	70	0.11	0.46	0.43	0.321	0.657	46	0.54	0.28	0.17	0.685	0.315
2000	Coast	Total	High	123	0.09	0.46	0.45	0.321	0.679	131	0.44	0.46	0.10	0.672	0.328
			Low	113	0.10	0.42	0.49	0.305	0.695	114	0.41	0.38	0.21	0.601	0.399
			Pooled	236	0.09	0.44	0.47	0.314	0.686	245	0.43	0.42	0.15	0.639	0.361
1993	Bay	Haffenreffer	High							31	0.39	0.42	0.19	0.597	0.403
1993	Bay	Haffenreffer	Low							7	0.29	0.71	0.00	0.643	0.357
1993	Bay	Barrington 1	High	43	0.12	0.35	0.53	0.291	0.709	44	0.43	0.43	0.14	0.648	0.352
1993	Bay	Barrington 1	Low	38	0.03	0.34	0.63	0.197	0.803	43	0.49	0.42	0.09	0.698	0.302
1993	Bay	Barrington 2	High	37	0.22	0.24	0.54	0.338	0.662	44	0.48	0.43	0.09	0.693	0.307
1993	Bay	Barrington 2	Low	41	0.15	0.44	0.41	0.366	0.634	44	0.59	0.36	0.05	0.773	0.227
1993	Bay	Totals	High	80	0.16	0.30	0.54	0.313	0.688	119	0.44	0.43	0.13	0.651	0.349
			Low	79	0.09	0.39	0.52	0.285	0.715	94	0.52	0.41	0.06	0.729	0.271
			Pooled	159	0.13	0.35	0.53	0.299	0.701	213	0.47	0.42	0.10	0.685	0.315
1993	Coast	Beavertail East	High							29	0.45	0.55	0.00	0.724	0.276
1993	Coast	Beavertail East	Low							29	0.48	0.52	0.00	0.741	0.259
1993	Coast	Beavertail West	High							25	0.44	0.40	0.16	0.640	0.360
1993	Coast	Beavertail West	Low							47	0.30	0.64	0.06	0.617	0.383
1993	Coast	Newport	High	40	0.18	0.40	0.43	0.375	0.625	44	0.52	0.36	0.11	0.705	0.295
1993	Coast	Newport	Low	44	0.11	0.50	0.39	0.364	0.636	43	0.53	0.37	0.09	0.721	0.279
1993	Coast	Sakonnet	High							43	0.44	0.40	0.16	0.640	0.360
1993	Coast	Totals	High							141	0.47	0.42	0.11	0.677	0.323
			Low							119	0.43	0.51	0.06	0.685	0.315
			Pooled	84	0.14	0.45	0.40	0.369	0.631	260	0.45	0.46	0.09	0.681	0.319

for all three markers using *G*-tests of independence (Sokal and Rohlf, 1981). Pooled data were also used to compare Bay vs. Coast and Maine vs. Rhode Island samples. To explore heterogeneity in genotype frequencies between High and Low microhabitats across multiple collecting localities we used log linear analyses (Lowry, 2002). To test hypotheses of genotype by environment interactions at different levels in the data set we used logistic regression analysis as implemented with the CATMOD procedure in SAS (SAS, 1996, version 6.12, run on a Dell Dimension 4100). This procedure uses a maximum likelihood approach to fit a model for categorical variables. We used a two-level ANOVA design where the independent categorical variables are microhabitat (High vs. Low tide height) and region (Maine vs. Rhode Island, or Bay vs. Coast). The dependent variable is genotype (observed numbers of SS, SF, FF individuals). Individual collecting localities are treated as replicates. The Rhode Island data are listed in the top part of Table 1 ("2000 Bay" samples) for Mpi and Gpi. The Maine data are the 1995 "Hot" locality samples reported in Table 1 of Schmidt and Rand (1999). These Maine samples are from estuary sites and are most comparable to Rhode Island "Bay" samples.

#### Enzyme activity analysis

Biochemical phenotypic differences between SS and FF electrophoretic genotypes were determined with an *in vitro* assay for Mpi activity. Individual barnacles of known genotype were homogenized in 100  $\mu$ l of distilled water, and then diluted to a total volume of 500  $\mu$ l. We determined total protein for each individual using BCA protein assay kits (Pierce, Rockford, Illinois). Individuals were then pooled randomly with three other individuals of the same genotype to a total protein concentration of 200  $\mu$ g/ $\mu$ l. Equal proportions of total protein came from all four individuals in each pool. This pooling is intended to randomize background genotype effects at other loci related to the biochemical assay. For example, the Mpi assay calls for Gpi in the reaction mix. By pooling multiple individuals, we establish a homogenate that carries one Mpi allozyme genotype, but multiple Gpi genotypes. Since Mpi and Gpi show no statistical linkage (Schmidt and Rand, 1999), this should randomize the effects of other loci (and substrates) that could confound assays based on individual homogenates. The pools of four individuals were constituted randomly with individuals of both High and Low microhabitats and thus represent enzyme assays from pooled individuals for which we report genotype frequencies in Table 1 (the 2,000 samples only).

The assay manipulated thermal stress and substrate availability. Pooled homogenates were incubated for 1 hr at one of four temperatures: 25°C, 37°C, 44°C, or 50°C. A reaction mix was prepared with 40  $\mu$ g/ $\mu$ l of incubated, pooled barnacle homogenate and 0.3 mM NADP, 1 U/ml PGI, 1 U/ml G6PD, 21  $\mu$ M DCIP, 3.3  $\mu$ M PMS, 20 mM Tris-HCl, pH 7.0 in each well of a

96-well microplate (see Clark and Wang, 1994). Mannose-6-phosphate (substrate) was added to each reaction well at one of four concentrations: 0 mM, 0.03 mM, 0.15 mM, and 0.75 mM. All assays were done in a Vmax kinetic 96 well microplate reader (Molecular Devices), reading every 10 sec for 4 min and 30 sec at 605 nm. Each 96-well plate contained a complete 4-substrate  $\times$  4-temperature block for each genotype (SS and FF) in order to compensate for random plate effects. Each of these combinations was replicated in three wells on each plate, and with three replicate plates from independent pools of homogenates. We analyzed four or five replicate homogenates for each region (Bay or Coast), with each homogenate replicated on two plates. In order to control for block (*i.e.*, plate) effects, enzyme activity for each well was reported as  $V_{\max(\text{well})} - V_{\max(\text{plate average})}$ . The effects of genotype, incubation temperature, substrate concentration, and region of sample origin (Bay vs. Coast) were tested with analysis of variance using JMP statistical package for Macintosh computers.

## RESULTS

### Habitat zonation at Mpi

Genotype and allele frequencies for Mpi and Gpi are reported in Table 1. Some patterns are evident at the genotype level, and others at the allele level; to aid in reading Table 1 we report genotype frequencies to two significant digits and allele frequencies to three digits. In summarizing the data here, we describe genotype patterns first, and then allele frequency patterns. Despite significant differences between some samples in allele and genotype frequencies, no significant departures from Hardy-Weinberg equilibrium (HWE) were detected for any samples after correcting for multiple tests, a pattern also observed in our Maine studies (Schmidt and Rand, 1999, 2001).

Using either *G*-tests of pairs of samples or log linear tests of pooled samples, there are no significant differences in Mpi genotype frequencies between High and Low microhabitats at various collecting localities in Narragansett Bay (data not shown). No difference between High and Low microhabitats are observed even when genotype counts for Bay and Coast sites are pooled. Based on our earlier studies of Maine localities, this result was not expected. A comparison of genotype frequencies at High and Low tide microhabitats from the Damariscotta River, Maine and Bay sites from Narragansett Bay, Rhode Island are presented in Figure 3. Mpi genotype frequency zonation is significantly different between Maine and Rhode Island. Logistic regression analysis of Mpi genotype counts shows a very significant interaction term between Region (Maine vs. Rhode Island) and Microhabitat (High vs. Low; see Table 2). This significant effect is evident in Figure 3, where the differences between High and Low microhabitats are opposite in direction for the SF and FF genotypes in the Maine and Rhode Island data.

Analysis of variance for temporal variation in

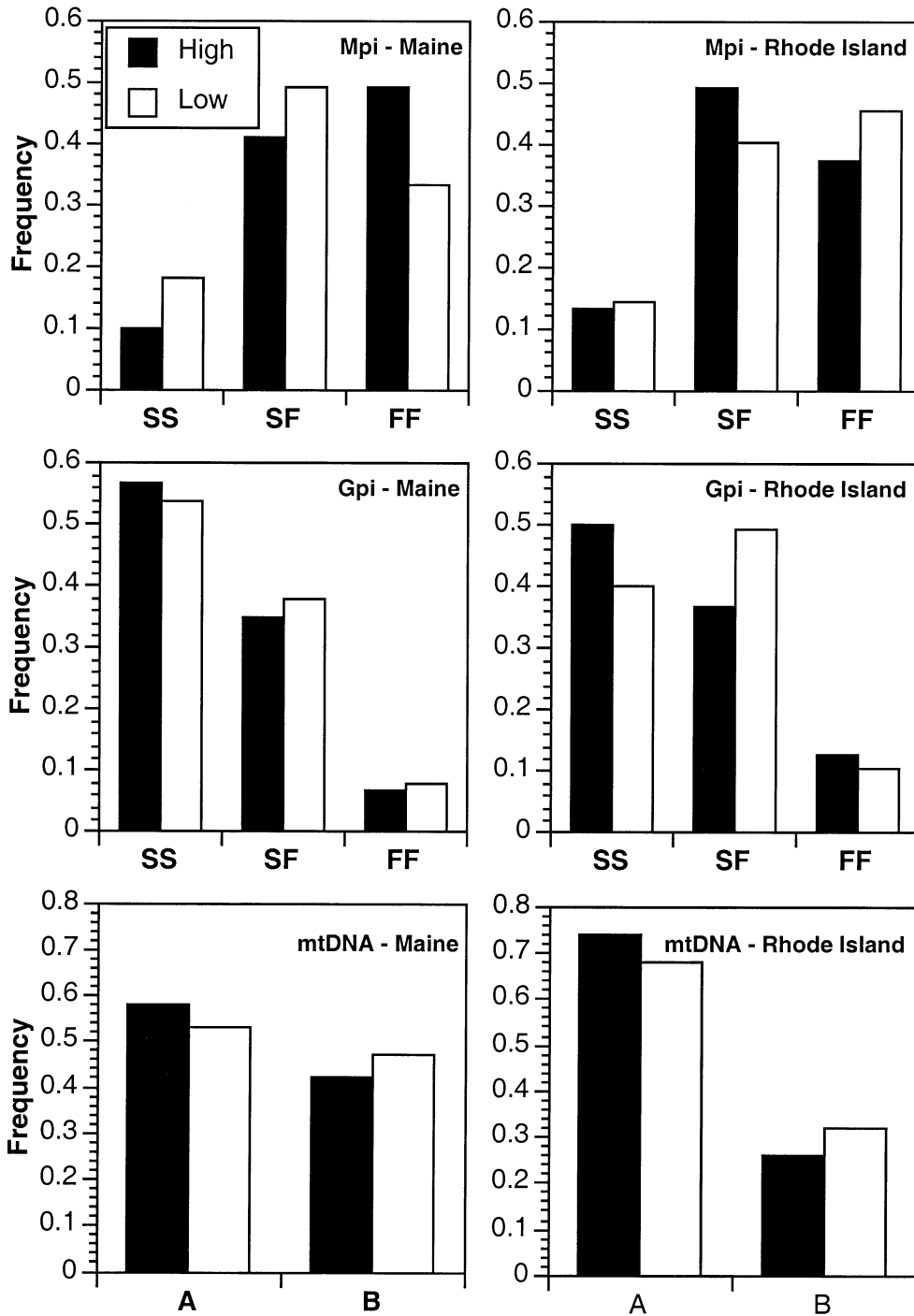


FIG. 3. Genotype frequencies at Mpi, Gpi and mtDNA in Maine and Rhode Island. In the Maine samples, Mpi High and Low frequencies are significantly different ( $P < 0.001$ ) but Gpi and mtDNA frequencies are not (data from Schmidt and Rand, 1999). Rhode Island data are from Table 1; Mpi and mtDNA High and Low frequencies are not significantly different, but the Gpi frequencies are ( $G = 8.23 < 0.01$ ). Rhode Island mtDNA data are from two Bay localities, as follows. Bristol Harbor High: 16 A, 6 B haplotypes; Low: 11 A, 4 B haplotypes. Jamestown Beach High: 23 A, 4 B haplotypes; Low: 14 A, 9 B haplotypes.

Rhode Island showed no significant Microhabitat by Year interaction term for either Mpi or Gpi (data not shown), indicating that the genotype zonation effects are statistically consistent in the 1993 and 2000 samples. There was a significant main effect of Year for Mpi Bay samples but this result pools across micro-

habitats is not relevant to the comparison between these intertidal microhabitats in Maine and Rhode Island.

Considering allele frequencies, the Mpi Slow allele is slightly more common in the High microhabitat than in the Low microhabitat at each of the four Bay sites

TABLE 2. Maximum likelihood ANOVAs for genotype-by-habitat interactions.\*

Source	Mpi		Gpi		mtDNA	
	df	Chi-Square	df	Chi-Square	df	Chi-Square
Intercept	2	170.05*****	2	202.71*****	1	17.37*****
Microhabitat	2	3.71	2	0.64	1	2.42
Region	2	0.43	2	10.58*****	1	11.30*****
Microhabitat **Region	2	16.48*****	2	10.31***	1	1.18
Likelihood ratio	20	13.74	20	14.54	6	3.47

\* Microhabitat refers to High vs. Low intertidal collecting sites. Region refers to Maine vs. Rhode Island. The data used are the 2000 Bay samples from Table 1 of this paper (Rhode Island), and the 1995 Hot localities (Maine) from Table 1 of Schmidt and Rand (1999).

\*\* =  $P < 0.05$ ; \*\*\* =  $P < 0.01$ ; \*\*\*\*\* =  $P < 0.0005$ ; \*\*\*\*\* =  $P < 0.0001$ .

in the Rhode Island 2000 sample, despite some heterogeneity among collecting localities in the genotype frequencies (see Table 1). This trend is not significant but it is opposite in direction from the pattern in Maine where Mpi Slow allele, and SS genotype frequencies, are consistently lower in High tide microhabitats (Schmidt and Rand, 1999). Pooled data from the Rhode Island 2000 sample show a significant difference in Mpi Slow allele frequencies between High Bay (38.0%) and Low Coast (30.5%) microhabitats ( $G = 3.88$ ;  $df = 1$ ,  $P < 0.05$ ), which represent the extremes of thermal and desiccation stress at the meso-scale in Rhode Island (~20 km). This Bay-Coast zonation of allele frequency across Narragansett Bay is consistent in direction with the allele zonation between High and Low microhabitats at individual Bay localities. This suggests that the selective effects of thermal stress operate at more than one spatial scale in Rhode Island, and are consistent in being opposite in direction from the zonation in Maine.

*Habitat zonation at Gpi*

Gpi shows significant genotype zonation between High and Low microhabitats in the Rhode Island Bay samples when data are pooled across collecting localities (Table 1;  $G$ -test:  $G = 8.3$ ,  $df = 2$ ,  $P < 0.025$ ; Log linear analysis of Microhabitat  $\times$  Genotype effect:  $G = 8.62$ ,  $df = 2$ ,  $P < 0.014$ ). A temporal comparison of the Rhode Island 2000 and 1993 data sets reveals no main effect of year, but a significant Microhabitat  $\times$  genotype term (log linear analysis:  $G = 6.32$ ,  $df = 2$ ,  $P < 0.05$ ) indicating that this High vs. Low zonation is consistent across years. In a two-level logistic regression analysis of genotype counts for Bay vs. Coast and High vs. Low, the interaction term is highly significant ( $P < 0.0098$ ). This indicates that Gpi genotype zonation is different in the Bay than on the Coast, which is evident from SF and FF counts in Table 1.

These patterns are in clear contrast to the data from Maine where Gpi behaves as a neutral marker with respect to High vs. Low microhabitats in data from over 12,000 barnacle genotypes (Schmidt and Rand, 1999, 2001; Schmidt *et al.*, 2000). Logistic regression analysis of Gpi genotype counts in Maine and Rhode Island shows a significant interaction term between Region (Maine vs. Rhode Island) and Microhabitat (High vs. Low; Table 2) confirming that Gpi responds

differently to microhabitat in Narragansett Bay compared to the Damariscotta River, Maine. This statistical effect is evident in Figure 3 where High and Low genotype frequencies are similar in Maine samples, but the SS and SF genotype frequencies differ by more than 10% between High and Low in Rhode Island.

Considering allele frequencies, a meso-scale pattern is evident at Gpi in the Rhode Island 2000 data. Pooled data from Bay vs. Coast sites reveal a significant allele frequency zonation between samples from the High Bay (Gpi Slow = 68.8%) and Low Coast (Gpi Slow = 60.1%; see Table 1;  $G = 5.48$ ,  $df = 1$ ,  $P < 0.05$ ). This allele frequency zonation is in the same direction as that observed between individual High and Low microhabitats at Bay Localities.

*Lack of mtDNA zonation*

No significant zonation is evident at mtDNA in the Bay samples from the Rhode Island 2000 collection, although data for some localities is missing (see Figure 3 legend). These data are consistent with our earlier observations that mtDNA behaves as a neutral marker (Brown *et al.*, 2001; Schmidt and Rand, 1999, 2001; Schmidt *et al.*, 2000). By inference from these inter-locus contrasts, Mpi and Gpi behave as non-neutral markers in Maine or Rhode Island. As shown in Figure 3, there is a clear difference between Maine and Rhode Island in the overall frequency of the A and B mtDNA haplotypes (see also Brown *et al.*, 2001). Logistic regression analysis shows that there is a significant effect of Region (Maine vs. Rhode Island), but there is no significant Region  $\times$  Microhabitat interaction (Table 2). Together these results confirm the difference in haplotype frequencies at this biogeographic scale, but indicate that the difference between High and Low microhabitats (*i.e.*, neutrality) is not significant between Maine and Rhode Island.

*Mpi activity*

In vitro enzyme activities for the Mpi-SS and Mpi-FF allozyme genotypes were examined in a factorial experiment manipulating temperature and substrate concentration. Pooled homogenates of each genotype were incubated for one hour at a prescribed temperature before determining activity. The amount of available substrate (mannose-6-phosphate) was simultaneously manipulated (see Materials and Methods).

TABLE 3. Analysis of variance for *Mpi* enzyme activity.\*

Source	df	Sum of squares	F ratio	P
Genotype	1	57.57	47.38	<0.0001
Temperature	3	329.90	90.51	<0.0001
Mannose-6-P concentration	3	342.59	93.99	<0.0001
Genotype × Temperature	3	33.59	9.22	<0.0001
Temperature × Mannose-6-P	9	91.50	8.37	<0.0001
Genotype × Mannose-6-P	3	20.31	5.57	0.0008
Genotype × Temperature × Mannose-6-P	9	18.87	1.73	0.0785
Model	31	915.88	24.32	
Error	1454	1766.52		

\* See Figure 4 for interaction effects. Genotypes are *Mpi*-SS vs. -FF homozygotes, Temperatures are one hour of pre-assay incubation at 25, 37, 44, or 50°C, and Mannose-6-P concentrations were assayed at four concentrations: no supplemental mannose-6-phosphate, 0.03 mM, 0.15 mM, and 0.75 mM.

Both the SS and FF homogenates show a significant decrease in enzyme activity following one hour of incubation at increased temperatures (see Fig. 4A). The SS genotype shows significantly greater activity than the FF genotype across all temperatures, but exhibits a greater proportional loss of activity across the temperature incubation gradient. Both the SS and FF genotypes show a significant increase in activity with increasing amounts of substrate added to the assay, but the SS genotype is more responsive to this increase in substrate (see Fig. 4B).

The significance of these effects is presented in three-way analysis of variance (Table 3) where the main effects are Genotype (SS vs. FF), Temperature (one hour at 25, 37, 44, or 50°C before running the assay), and mannose-6-phosphate concentration in the assay (no additional mannose-6-phosphate added, 0.03 mM, 0.15 mM, or 0.75mM). All possible interaction terms are presented as well, and all terms except the three-way interaction are highly significant. The strong effects of temperature and mannose-6-phosphate concentration are expected given the range of experimental conditions. The important biological observations from this ANOVA are that 1) genotype has a strong main effect (6.3% of model sum of squares), 2) there is a strong Genotype by Temperature interaction, and 3) there is a strong Genotype by mannose-6-phosphate concentration interaction (3.7%, and 2.2% of Model sum of squares, respectively). While it is difficult to translate *in vitro* enzyme activity of total homogenates into fitness effects in the field, these results show that there are biochemical phenotypic differences between the alternative homozygotes at *Mpi*, and that these genotypes respond differently to temperature and substrate effects.

#### DISCUSSION

Two important aspects of multiple-niche models for the maintenance of genetic variation are the spatial scale of the fitness variation and the proportion of the various niches that determine average fitness (Levene, 1953). If the environment is partitioned into discrete coarse-grained niches, the opportunity for selective maintenance of polymorphism is greatest (Maynard Smith and Hoekstra, 1980). Our previous work has

provided evidence for balancing selection on the *Mpi* polymorphism consistent with the Levene (1953) model. This is based on static patterns of genotype zonation (Schmidt and Rand, 1999), experimental transplants of individuals in the field (Schmidt *et al.*, 2000) and the lab (Schmidt, 2001), and survivorship analyses of cohorts through time (Schmidt and Rand, 2001). These studies have shown that environmental grain size on the scale of 1–10 m among discrete intertidal microhabitats in the bays and estuaries of the Maine coast is important in balancing selection. However, pelagic larval dispersal in *Semibalanus balanoides* leads to extensive gene flow. Analyses of mtDNA variation indicate that bay and coastal sites separated by more than 20 km are effectively panmictic ( $N_e m > 100$ ) and gene flow between Maine and Rhode Island does occur at low levels (see Brown *et al.*, 2001). Any environmental variation that lies within this range of effective gene flow could contribute to the maintenance of polymorphism suggesting that balancing selection may operate on multiple spatial scales.

#### Geographic variation in selection at *Mpi*

Here we show that *Mpi* genotype and allele frequency zonation between High and Low-tide microhabitats is opposite in direction in Narragansett Bay, Rhode Island compared to the Damariscotta River, Maine. This difference is due to a reversal in the zonation of SF and FF genotypes in Maine vs. Rhode Island (Table 1 and Fig. 3). How do these results affect our earlier interpretation of balancing selection at *Mpi*? In Maine, the evidence for balancing selection under the Levene (1953) model comes from the observation that *Mpi* FF genotypes have highest fitness at the “hot” extreme, and *Mpi*-SS genotypes have highest fitness at the “cool” extreme of the intertidal thermal gradient. Moreover, these fitness differentials are intermediate in intermediate microhabitats. Stability analyses of the fitness data were modeled using two niches in roughly equal proportions (Schmidt *et al.*, 2000; Schmidt and Rand, 2001). The real world is rarely partitioned into such a simple dichotomy. As considered in the Levene model and its derivatives (*e.g.*, Dempster, 1955; Gillespie, 1976), the key issue is the harmonic mean fitness of the various genotypes

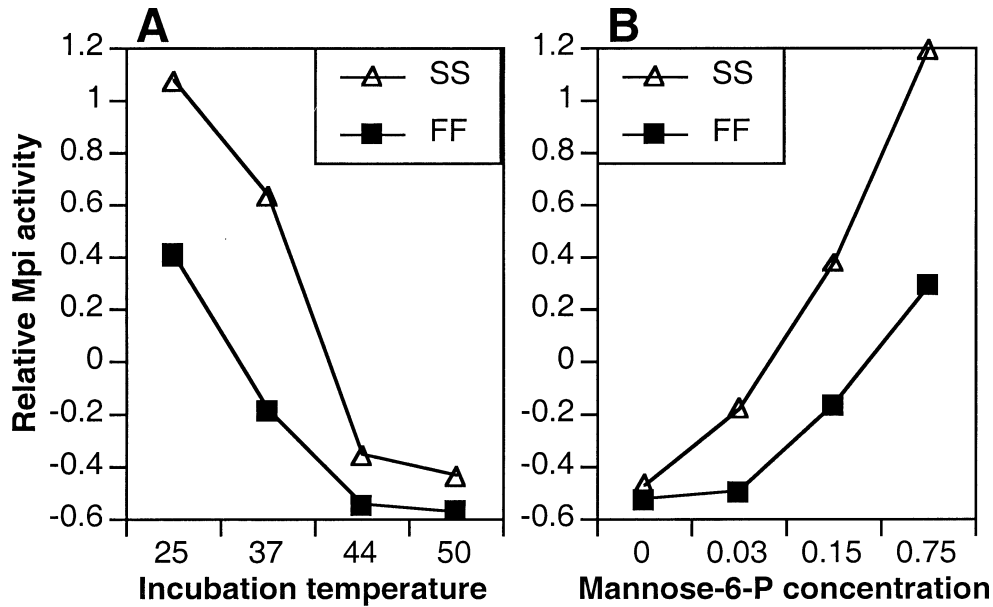


FIG. 4. Enzyme activities of Mpi-SS and -FF genotypes. A. Effect of temperature on enzyme activity. Barnacle homogenate was incubated for one hour at the temperature specified on the X-axis prior to performing the assay. B. Effect of substrate (mannose-6-phosphate) concentration on enzyme activity. Substrate was added to the homogenate at four different final concentrations as specified on the X-axis. Differences between genotypes, temperatures and substrate concentrations are highly significant (see Table 3).

at a locus weighted by the relative proportions of the various microhabitats in nature. The apparent change of sign in selection coefficients for Mpi genotypes on the a scale of 100's of kilometers considered in this study does not undermine the Levene-like selection that has been documented at a scale of 10's of meters in Maine. Indeed, the likelihood of maintaining a polymorphism can be increased by limited gene flow between niches (Karlín, 1982). The data presented here indicates that Maine and Rhode Island are distinct niches with respect to selection, and gene flow between these regions is dramatically reduced relative to gene flow between sites within a given bay (Brown *et al.*, 2001). This balance between gene flow and selection at a broad spatial scale can only contribute to balancing selection acting at a small spatial scale among intertidal microhabitats.

A recent study in Canada confirms that the Mpi polymorphism shows additional spatial variation in apparent selection. Samples of *S. balanoides* spanning the mouth of the Miramichi estuary in the Gulf of St. Lawrence show a strong decrease in the frequency of the Mpi-F allele in southern (presumably warmer) collecting localities, and contrasts with putatively neutral microsatellite markers implicate selection at Mpi along the collecting transect (Dufresne *et al.*, 2002). Explicit comparisons between High and Low intertidal habitats were not described in Canadian samples so it is difficult to establish a clear comparison. Nevertheless, the data suggest that a simple clinal model of selection on Mpi is rejected in favor of local selection between habitats at both micro and meso scales across the range of *S. balanoides*. The thermal profiles of the substrate, air and water can vary greatly at micro scales through-

out the intertidal (*e.g.*, Helmuth and Hoffmann, 2001), and additional thermal data are needed in both Canada and Rhode Island to clarify these biogeographic patterns.

*Geographic variation in selection at Gpi*

The data presented here show a significant microhabitat zonation at Gpi in Rhode Island while this locus is neutral to intertidal microhabitats in Maine (Schmidt and Rand, 1999, 2001; Schmidt *et al.*, 2000). This difference in Gpi genotype zonation between High and Low microhabitats is itself significantly different between Maine and Rhode Island (see Fig. 3 and Table 2). Dufresne *et al.* (2002) report that Gpi also shows a subtle but significant change in allele frequency across the Miramichi estuary while most microsatellites do not show such a change. Neutrality at Gpi among microhabitats in Maine and selective zonation in Rhode Island (and the Gulf of St. Lawrence) are not sufficient conditions for a Levene model of balancing selection at a macro-geographic scale. However, there is a significant difference in the microhabitat zonation at Gpi between Bay and Coast sites in Narragansett Bay. Logistic regression analyses show a significant ( $P < 0.0098$ ) interaction between Region (Bay vs. Coast) and Microhabitat (High vs. Low) in Rhode Island samples. The shifts in the inferred fitnesses of the SF and FF Gpi genotypes between Bay and Coast suggest that balancing selection at Gpi may operate at the meso scale (~10s of kilometers). While experimental manipulations are needed to address the issue of balancing selection at Gpi in barnacles, the data presented here, and those of Holm and Bourget (1994), Dufresne *et al.* (2002) and Schmidt and Rand

(1999, 2001) do establish the prerequisite that selection among Gpi genotypes varies geographically.

MtDNA shows no significant microhabitat zonation in either Maine or Rhode Island, and no difference in zonation between these regions, consistent with our earlier evidence that mtDNA acts as a neutral marker. This strongly implicates selection at or near the enzyme loci (cf. Berry and Kreitman, 1993; Schmidt and Rand, 1999).

#### *Thermal stress, diet and hidden adaptive potential*

What might be the source of this geographic variation in selection at Mpi and Gpi? Variation in thermal stress is a likely candidate given the strong evidence for thermal effects on enzyme polymorphisms in other invertebrates (Mpi in Amphipods; McDonald, 1991; Gpi in butterflies; Watt, 1977). In Rhode Island summertime substrate temperatures in the marine intertidal are up to 10°C warmer than those in Maine (Bertness and Gaines, 1993). But the contrasting directions of clinal patterns within Maine, Rhode Island and Canada clearly show that temperature is not the only important environmental factor in governing selection at Mpi and Gpi. The enzyme activity data reported here show a significant interaction between temperature and mannose-6-phosphate concentration. The elegant experiments by Schmidt (2001) show that the Mpi polymorphism is governed by an interaction between the availability of dietary sugars and thermal (or desiccation) stress.

When Maine barnacles are grown in the laboratory with supplemental mannose (not mannose-6-phosphate), growth rates are significantly higher than a no-sugar control under ambient temperatures. However, when barnacles experience thermal stress under conditions of supplemental mannose, growth rates are significantly reduced relative to controls (Schmidt, 2001). Importantly, under thermal stress and supplemental mannose, there are significant differences in growth rates among the genotypes (the Mpi-FF genotype grows the fastest growth, SF is intermediate and SS is slowest). This effect may be the result of a net decrease in ATP pools. Hexoses are rapidly phosphorylated by hexokinases, converting ATP to ADP. The apparent thermal lability of Mpi could result in the loss of ATP regeneration due to reduced carbohydrate metabolism downstream from Mpi. For example, supplemental mannose is metabolically toxic to honeybees due to low Mpi activity and depleted ATP pools (Saunders *et al.*, 1969). A comparable effect may be operating among Mpi genotypes in barnacles. There are many additional factors that need to be examined to determine if this temperature-lability/ATP-recovery hypothesis holds. We do not know if the activities of these enzymes fluctuate during the tidal cycle in concert with food or oxygen availability. Barnacles may shift between oxidative phosphorylation and the lactate pathway of ATP production in response to fluctuation in oxygen levels (Hochachka and Somero, 2002). Moreover, during high tide when the opercular

valves are closed, temperature, desiccation, and oxidative stress co-vary. And heat shock proteins may be important modulators of Mpi and Gpi activities during (and after) high tide stress (see Hofmann, 2002 and Tomanek, 2002 in this issue).

Our current working hypothesis regarding the patterns of Mpi and Gpi zonation in Maine and Rhode Island invokes an interaction between thermal stress and variation in dietary carbohydrates. As filter feeders, barnacles consume phytoplankton, algae and presumably a wide diversity of organisms of the appropriate particle size. Various phytoplankton species have high concentrations of mannose polysaccharides (McDonald, 1991), and mannose and mannitol is abundant in the cell walls of algae (Kreger, 1962). Moreover, barnacles have the enzyme activity to digest complex carbohydrates into simple mannose (Molodsov *et al.*, 1974). The phytoplankton composition of Narragansett Bay, Rhode Island and estuaries in the Gulf of Maine are different (*e.g.*, Hargraves, 1988; Wong and Townsend, 1999). We do not know if the carbohydrate intake of barnacles differs as a result, but this remains a testable hypothesis. With different Mpi genotypes showing activity differences as a function of temperature and substrate concentration (Fig. 4), geographic variation in microhabitat zonation seems likely. We do not have similar activity data for Gpi, but comparable thermal effects have been reported for other crustaceans (Patarrello and Battaglia, 1992).

In insects there is considerable evidence for adaptive variation at Gpi (*e.g.*, Watt, 1977; Dahlhoff and Rank, 2000), but a virtual absence of such evidence for Mpi. In marine invertebrates, Mpi is the most consistently polymorphic enzyme and shows the highest heterozygosity of enzymes of central metabolism, but Gpi shows similar patterns (Hedgecock *et al.*, 1982). Presumably glucose and Gpi polymorphisms are more important to adaptive potential of nectar- or cellulose-feeding insects, while mannose and Mpi polymorphisms are of greater adaptive potential in filter-feeding marine invertebrates that consume mannose-rich polysaccharides. Similar shifts in the relative adaptive importance of Mpi and Gpi may well be going on at a much finer phylogenetic scale within *S. balanoides*. This genotype-diet-thermal stress hypothesis predicts that glucose is a more significant component of the barnacle diet in Rhode Island, where Gpi is non-neutral, while mannose is a more important component of the diet in Maine, where Mpi is more strongly non-neutral. These parallel feeder pathways to glycolysis may provide considerable flexibility in adapting to heterogeneous environments (see Fig. 1). This hypothesis can be tested with chemical analyses of barnacle diets in these two regions, and with genotype-specific growth analyses and enzyme activities under supplemental mannose, glucose, or appropriate phytoplankton species.

Many of the predictions of the genotype-by-environment hypothesis proposed here may need to be revised when complete DNA sequence data are obtained

for Mpi and Gpi in *S. balanoides*. It may be that the electrophoretic alleles and genotypes mask cryptic differences at the DNA level, as is evident for allozyme loci in *Drosophila* (e.g., Verrelli and Eanes, 2001). With comparable nucleotide data for Mpi and Gpi in barnacles, the zonation of genotypes across intertidal microhabitats will provide a powerful ecological assay for potential epistatic interactions among specific nucleotide polymorphisms that underlie the Fast and Slow electromorphs at each locus. Coupled with field and laboratory manipulations of growth rates and enzyme activities, these studies promise to provide a complete picture of the molecular, physiological and ecological factors that promote the adaptation to heterogeneous environments.

## ACKNOWLEDGMENTS

We would like to thank Mark Bertness for advice on field work in Maine and Rhode Island. Adam Fry and two anonymous reviewers provided helpful comments on the manuscript that greatly clarified a number of points. Lea Sheldahl provided general assistance in the lab. Supported by NSF grants DEB 9527709 and DEB 010850 to DMR.

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