The effects of diet and physiological stress on the evolutionary dynamics of an enzyme polymorphism

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In the northern acorn barnacle *Semibalanus balanoides*, polymorphism at the mannose-6-phosphate isomerase (*Mpi*) locus appears to be maintained by distinct selection regimes that vary between intertidal microhabitats. The goal of the present experiment was to elucidate the mechanism of selection at the *Mpi* locus by examining the relationship between genotype and fitness-related life-history traits in laboratory manipulations. When barnacles were cultured on a mannose-supplemented diet and exposed to thermal stress, different *Mpi* genotypes exhibited differences in the rate of growth that predicted survivorship. In contrast, no such relationship was observed in control or fructose-supplemented dietary treatments either in the presence or in the absence of stress. Similarly, the phenotype and survivorship of genotypes at another allozyme locus and a presumably neutral mitochondrial DNA marker were homogeneous across all treatments and unaffected by experimental manipulations. These results suggest that the differential survivorship of *Mpi* genotypes in the field and laboratory results from a differential ability to process mannose-6-phosphate through glycolysis. The widespread polymorphism at *Mpi* observed in marine taxa may reflect the interaction between dietary composition and environmental heterogeneity in intertidal habitats.

Keywords: balancing selection; mannose; *Mpi*; *Semibalanus balanoides*

1. INTRODUCTION

Determining how selection operates on particular polymorphisms is essential to understanding the importance of genetic variation in natural populations (Gillespie 1991). Four basic criteria must be met in order to establish the direct action of selection at a particular gene: first, genetic variants must possess functional differences that influence phenotype; second, genotype-dependent differences in phenotype must predictably affect some aspect of organismal performance; third, differential performance must result in fitness differences between genotypes; and, fourth, experimental results must explain patterns of genotype-frequency variation in natural populations (Clarke 1975; Koehn 1978; Feder & Watt 1992). Comprehensive analyses of selection at allozyme loci remain limited to a small number of loci and species (e.g. Burton & Feldman 1983; DiMichele & Powers 1982; Hilbish & Koehn 1985; Watt *et al*. 1983). However, such studies have provided valuable insights into the dynamics of selection at the genic level and the potential role of molecular variation in the adaptation of organisms to heterogeneous environments.

This study examines the mechanism of selection at the mannose-6-phosphate isomerase (*Mpi*, EC 5.3.1.8) allozyme locus in the northern acorn barnacle, *Semibalanus balanoides*. Previous studies have established that *Mpi* genotype frequencies are predicted by the temperature and/or level of desiccation stress experienced by barnacles in a given intertidal habitat (Schmidt & Rand 1999). Genotype frequencies for another allozyme locus (glucose-6-phosphate isomerase, *Gpi*, EC 5.3.1.9) and a presumably neutral mitochondrial (mtDNA) marker have

been found to be homogeneous across habitats. Experimental manipulations in the field have revealed that such patterns result from the differential mortality of *Mpi* genotypes (Schmidt *et al*. 2000), which occurs at a specific life-history stage (P. S. Schmidt, unpublished data). Furthermore, the empirical data from this body of work demonstrated a remarkable fit to the Levene (1953) model of balancing selection. This supports the hypothesis that the *Mpi* polymorphism is actively maintained by selection and represents an adaptation to the heterogeneous intertidal environment.

These studies do not unambiguously demonstrate selection at the *Mpi* locus, or suggest why genotype at *Mpi* influences survivorship in various intertidal environments. McDonald (1991) postulated that selection at this locus in marine invertebrates may result from a combination of high levels of dietary mannose and related compounds and exposure to variable environments. Here, this hypothesis is tested in an intertidal barnacle by examining the effects of diet and temperature/desiccation stress on organismal growth and the survivorship of genotypes for three genetic markers.

2. MATERIAL AND METHODS

(a) *Experimental design*

Barnacle larvae were collected on granite settlement plates ($n = 85$) that were bolted to the substrate in the low intertidal zone. The plates were deployed at a site characterized by high annual settlement and minimal larval mortality (Glidden Ledge, Damariscotta River Estuary, ME, USA, 44°54′ N, 69°35′ W; Leonard *et al*. 1998). Larvae were collected over a period of seven days and then transported to the laboratory.

Each plate was randomly assigned to a dietary treatment: fructose addition (F), mannose addition (M) and a control treatment in which no dietary supplement was added (C). Barnacles were kept in 30-l aquaria, which were immersed in a

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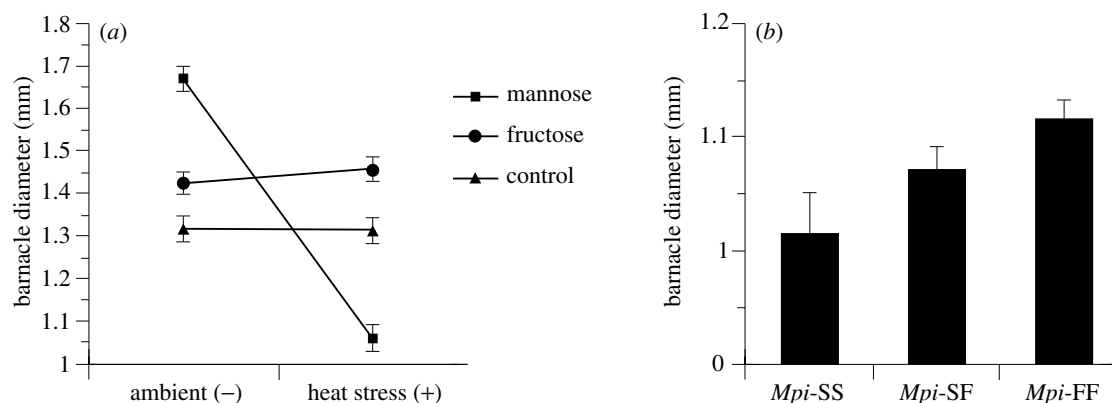
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Figure 1. (*a*) The effect of the interaction between diet and temperature on barnacle growth. The effect of mannose was dependent on temperature treatment. In contrast, barnacle size was equivalent between stress-level treatments for both the fructose and the control diets. (*b*) Mean sizes of *Mpi* genotypes in the M+ treatment combination. *Mpi*-FF homozygotes were significantly larger than *Mpi*-SS genotypes.

flowing seawater table to maintain water temperature at a natural level. Water pumps/aerators were used to generate flow and maintain oxygen levels. In the two sugar-addition treatments, barnacles were cultured on a 1% w/v solution of D-sugar (*ca.* 60 mM). To replenish sugars and reduce the concentration of waste products in each aquarium, 2 l of water were removed and 2 l of sugar–seawater at 1% w/v were added each day. In the control treatments, 30 l of plain seawater was added initially and 2 l replaced each day.

Each plate was also randomly assigned to one of two stress-level treatments, either high ($+$) or ambient ($-$) temperature. Thus, there were six treatment combinations: mannose diet–high temperature (M$+$), mannose diet–ambient temperature (M$-$), fructose diet–high temperature (F$+$), fructose diet–ambient temperature (F$-$), control diet–high temperature (C$+$) and control diet–ambient temperature (C$-$). Over the course of the experiment, a natural 12 h tidal cycle was simulated. During the 'daytime low tide', plates assigned to the high-temperature treatment were removed from the tanks and heated to a maximum temperature of 32 °C using 200 W heat lamps. Temperature data loggers (Onset Computer Corporation, Pocasset, MA, USA) were used to ensure thermal homogeneity across replicate plates. During the 'night-time low tide', plates in the high-temperature treatment were not exposed to the heat lamps. In the ambient-temperature treatment, plates were also removed from the tanks during both simulated low tides but were not exposed to heat stress. Barnacles were cultured in the laboratory for a total of 19 days.

(b) Life-history trait measurements and genotype determination

Two randomly selected plates in each treatment combination were examined under a dissecting microscope every two days to monitor mortality. After an approximate mean of 60% mortality was observed in at least one treatment, percentage mortality was recorded for all plates. This value was chosen to reflect patterns of mortality previously observed in thermally stressful intertidal habitats (P. S. Schmidt, unpublished data). Approximately 200 individuals were subsequently collected from each replicate plate and stored at -80 °C until used in the genetic analyses.

The increase in barnacle diameter was used as an estimate of organismal growth. In each of the six treatment combinations,

Table 1. *Four-factor ANOVA for barnacle diameter* (*mm*)

(All factors were fixed effects and the error mean square was used as the denominator in calculating *F* ratios.)

source	d.f.	ss	*F*	*p*
diet	2	2.175	13.101	0.0001
temperature	1	7.302	87.986	0.0001
Mpi genotype	2	0.580	3.492	0.0309
Gpi genotype	2	0.080	0.480	0.6190
diet × temperature	2	19.104	115.010	0.0001
error	812	67.389	—	—

approximately 150 individuals were measured using an ocular micrometer on a dissecting microscope. Diameter was recorded across the middle of the operculum, parallel to the suture between the tergal and scutal plates. Barnacles were removed from the freezer, diameters were recorded and then the samples were genotyped.

The genotype of each individual was determined according to the protocols outlined in Schmidt & Rand (1999) for three genetic markers: the *Gpi* and *Mpi* allozyme loci and a 401 bp fragment of the mtDNA control region. Allozyme genotype was determined using cellulose acetate electrophoresis (Hebert & Beaton 1989). Each locus has two common alleles designated according to relative mobility, F for the fast allele and S for the slow allele. Polymerase chain reaction (PCR) was used to amplify the mtDNA control region; the forward primer was *Iso2* from Simon *et al.* (1994) and the reverse primer was designed from *S. balanoides* sequence near the 5′-end of the 12sRNA gene (Brown 1995). A *Dde*-I restriction-enzyme polymorphism defined two mtDNA haplotypes (A and B) that were visualized on 3% agarose gels. The PCR fragment was used as a presumably neutral marker (Brown 1995) in comparisons with the two allozyme loci.

Genotype frequencies were determined for the following: six plates that were sampled prior to the initiation of the experiment (to generate pre-selection estimates); six plates from each of the high-temperature treatments (M$+$, F$+$, C$+$) and two plates from each of the ambient-temperature treatments (M$-$, F$-$, C$-$). No mortality was observed in the ambient-temperature treatments but two replicates were processed to ensure that

Table 2. *Observed genotype frequencies*

(Mean frequencies are given for each treatment.)

| diet	stress	Mpi				Gpi				mtDNA		
		n	SS	SF	FF	n	SS	SF	FF	n	A	B
pre-selection	−	437	0.157	0.478	0.364	421	0.518	0.382	0.100	374	0.529	0.471
mannose	−	156	0.143	0.566	0.291	159	0.518	0.421	0.061	95	0.543	0.457
fructose	−	142	0.148	0.479	0.373	154	0.579	0.356	0.065	72	0.515	0.485
control	−	166	0.156	0.499	0.345	143	0.507	0.398	0.095	73	0.462	0.538
mannose	+	419	0.084	0.453	0.463	421	0.520	0.413	0.066	321	0.494	0.506
fructose	+	418	0.166	0.516	0.318	430	0.530	0.398	0.072	303	0.522	0.478
control	+	413	0.150	0.521	0.329	413	0.508	0.422	0.070	301	0.483	0.517
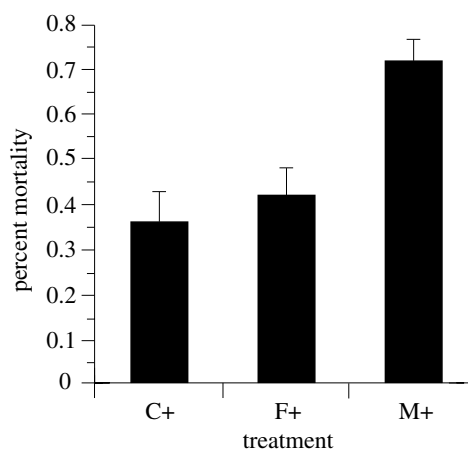
Figure 2. Percentage mortality in the high-temperature treatment combinations. Mortality was higher in the M+ treatment than in either the F+ or the C+ treatments, in which barnacles exhibited similar patterns of survivorship.

genotype frequencies were similar to those of the pre-selection samples.

(c) *Statistical analysis*

Analysis of variance (ANOVA; JMPIN, v. 3.2.6, SAS Institute 1999) was used to determine treatment effects on arcsine square-root transformed percentage mortality. The effects of diet and temperature treatments, the interaction term and *Mpi/Gpi* genotype on barnacle size were also examined with ANOVA. It was of particular interest to examine size differences between the two homozygous *Mpi* genotypes in the M+ and M− treatment combinations. Within-model planned comparisons were performed using *t*-tests, adjusting probability values for two comparisons.

The procedure CATMOD (SAS, v. 6.12, SAS Institute 1998; Stokes *et al.* 1995) modelled the relationship between genotype and the predictor variables. No mortality was observed in any of the ambient-temperature treatments (M−, F−, C−), which were subsequently not included in the analysis. Therefore, the sole predictor variable used in the regressions was a combination of diet and temperature treatments (M+, F+, C+). A separate analysis was performed for each of the three genetic markers. The CATMOD procedure employs maximum-likelihood estimation of generalized logits. For *Mpi* and *Gpi*, which have three genotypes, two logits were formed:

$$logit_{h1} = \log(\pi_{h_1}/\pi_{h_3}), \tag{1}$$

$$logit_{h2} = \log(\pi_{h_2}/\pi_{h_3}), \tag{2}$$

where π_{h1} is the probability of being genotype 1 in the *h*th sub-population and $h = 1$, 2 and 3 for the treatment combinations M+, F+ and C+, respectively. Only one logit was formed for the mtDNA control-region data as there were two haplotypes. In each analysis, a backward stepwise approach was used to select final regression models. In each regression, genotype counts from the initial (pre-selection) samples were also included as a reference treatment. This allowed odds-ratio comparisons between treatments as well as between post- and pre-selection samples. Thus, the regressions modelled the effects of each treatment combination on genotypic probabilities as well as how these probabilities changed over the course of the experiment.

3. RESULTS

(a) *Barnacle growth*

Diet had a significant effect on barnacle growth, as did the diet–temperature interaction term (table 1). The significance of the latter is almost entirely due to the difference in mean diameter between the M+ and M− treatment combinations (figure 1*a*). In the high-temperature treatment, the mean diameter of individuals in the M dietary treatment was substantially smaller than in the other two treatments, but in the absence of heat stress it was substantially greater. Mannose was a preferred substrate in the absence of thermal stress but retarded growth when individuals were exposed to higher temperatures.

Genotype at the *Gpi* locus did not explain a significant amount of the variation in diameter across all treatment combinations; however, *Mpi* genotype had a significant effect (table 1). In each treatment combination, the rank order of *Mpi* genotypic sizes was consistent: the mean size of the *Mpi*-FF genotype was greater than that of the *Mpi*-SF genotype, which was greater than that of the *Mpi*-SS genotype. In the M− treatment, the mean diameters of the *Mpi*-FF and *Mpi*-SS genotypes were not significantly different (two-tailed test, $p > 0.14$). However, this comparison was significant in the M+ treatment combination (two-tailed test, $p < 0.02$; figure 1*b*). The combination of dietary mannose supplementation and

Table 3. *Maximum-likelihood parameter estimates for the two logits modelled in the* Mpi *regression*

variable	interpretation	para-meter	estimate	s.e.m.	Wald χ^2	p	para-meter	estimate	s.e.m.	Wald χ^2	p
			logit$_1$ (FF/SS)					logit$_2$ (SF/SS)			
intercept	average generalized logit	a1	1.0048	0.0731	188.97	0.0001	a2	1.3111	0.0703	348.18	0.0001
treatment	differential effect for M +	b1	0.6245	0.1315	22.54	0.0001	b2	0.3103	0.1301	5.69	0.0170
	differential effect for F +	b3	−0.2784	0.1240	5.04	0.0247	b4	−0.0506	0.1167	0.19	0.6643
	differential effect for C +	b5	−0.1957	0.1311	2.23	0.1356	b6	−0.0513	0.1243	0.17	0.6798

Table 4. *Odds ratios calculated from the* Mpi *regression*

(PS, pre-selection samples.)

comparison	M + /F +	M + /C +	M + /PS	F + /C +	F + /PS	C + /PS
logit$_1$ (SS/FF)	2.47[a]	2.27[a]	2.17[a]	0.92	0.88	0.96
logit$_2$ (SF/FF)	1.44	1.44	1.68[a]	1.00	1.17	1.17

[a] 95% confidence intervals do not include 1.0.

exposure to physiological stress resulted in *Mpi*-genotype-specific differences in growth.

(b) Survivorship

Percentage mortality in the ambient-temperature treatment combinations was effectively zero. This lack of mortality, and the larger size of individuals in the M− and F− treatments compared to the control treatment, suggests that neither fructose nor mannose by itself was toxic to barnacles. Within the high-temperature treatment, diet significantly affected survivorship (one-way ANOVA on arcsine square-root transformed percentage mortality, $F = 10.25$, $p < 0.001$). Figure 2 shows that mortality was elevated in the M + treatment combination relative to the F + and C + treatment combinations. Thermal stress alone resulted in extensive mortality and this effect was magnified when mannose was supplemented in the barnacles' diet.

(c) Differential survivorship of genotypes

In the regression analyses for both the *Gpi* and mtDNA control-region loci, the sole predictor variable (treatment combination) could be dropped without compromising the fit of the model to the observed genotype counts. Model fit was evaluated by the likelihood ratio goodness-of-fit test (LR), for which a p-value greater than 0.05 indicates a satisfactory fit of the regression model to the observed data (*Gpi*, LR $\chi^2 = 33.38$, d.f. = 46, $p > 0.8$l; mtDNA, LR $\chi^2 = 8.56$, d.f. = 23, $p > 0.97$). In other words, the average generalized logit across all treatment combinations, the intercept term, was a sufficient predictor of genotypic probabilities in all treatment combinations. Treatment had no effect on *Gpi* genotype or mtDNA haplotype frequencies. In addition, no significant change between pre- and post-selection samples was evident (table 2).

In contrast, *Mpi* demonstrated a clear non-neutral pattern. The M + treatment combination had a significantly positive effect on both logits (table 3). The frequency of the *Mpi*-FF genotype was elevated, whereas the frequency of the *Mpi*-SS genotype was reduced, in the M+ treatment combination relative to other samples (table 2). The significant and negative differential effect of the F + treatment combination on logit$_1$ (table 3) was somewhat surprising and is similar to previous results obtained for barnacles associated with the alga *Ascophyllum nodosum* (Schmidt & Rand 1999; Schmidt *et al*. 2000). In contrast to the two sugar-addition–high-temperature treatments, the C + treatment did not have a significant effect on either logit.

The odds of sampling the *Mpi*-FF rather than the *Mpi*-SS genotype (odds ratio for logit$_1$) were significantly higher in the M + treatment than in the F + or C + treatments or the pre-selection samples (table 4). Not only were the genotype frequencies variable across treatments but they changed significantly in the M + treatment during the experimental period. Similarly, the odds of a barnacle in the M + treatment being the *Mpi*-SF genotype rather than the *Mpi*-SS genotype (logit$_2$) were higher than in the pre-selection samples (table 4). The regression parameters (table 3) showed that the F + treatment had a negative effect on logit$_1$, indicating a decrease in the frequency of the *Mpi*-FF genotype and/or an increase in the frequency of the *Mpi*-SS genotype. However, the odds ratios for the F + treatment compared to the C + treatment and the pre-selection samples were not significantly different from 1.0 (table 4).

4. DISCUSSION

The combination of dietary mannose and temperature and/or desiccation stress had a large non-additive effect on

two fitness components: growth and survivorship. At ambient temperatures, barnacles grew faster and larger on mannose compared to the fructose and control treatments. When exposed to high temperatures, however, growth was drastically reduced and mortality elevated in comparison to the other two treatments. Similarly, neither the presence of dietary mannose nor exposure to stress alone resulted in the differential mortality of genotypes. Only when the two factors were combined did *Mpi* genotypes exhibit significant differences in growth and survivorship.

The experimental results suggest a causative link between *Mpi* genotype and fitness under various environmental conditions. First, the contrasting responses of the three genetic markers implicate selection at the *Mpi* locus (Berry & Kreitman 1993; Karl & Avise 1992; Pogson *et al.* 1995). Second, the pronounced effects of mannose relative to fructose and control treatments are consistent with the hypothesis that selection operates at *Mpi* in barnacle populations. In glycolysis, the metabolism of fructose and mannose differs by a crucial enzymatic step: the action of MPI. Both sugars are initially phosphorylated by hexokinase, although in this species it is unknown whether each substrate is catalysed by the same enzyme. Fructose-6-phosphate can then directly enter the next step in glycolysis, whereas mannose-6-phosphate must first be isomerized by MPI to fructose-6-phosphate.

In contrast to terrestrial autotrophs, in marine algae and phytoplankton mannose and related compounds may be present in relatively high concentrations (e.g. Craigie 1974; Kreger 1962). Additionally, some barnacle species possess the enzymes needed to convert complex mannose-containing compounds to simple sugars (Molodsov *et al.* 1974). Although the specific chemical composition of the barnacle diet is unknown, mannose may comprise a non-negligible component for *S. balanoides* and other marine invertebrates. Catalysis by MPI may be a critical step in barnacle intermediary metabolism, similar to the importance of GPI in the metabolism of glucose in other taxa (e.g. Watt *et al.* 1983).

MPI activity affects survivorship in other organisms. The combination of low MPI activity and the presence of dietary mannose can deplete adenosine triphosphate (ATP) reserves and cause mortality (e.g. Arnold *et al.* 1974; De la Fuente *et al.* 1986; Herold & Lewis 1977). Provided there is no feedback regulation of hexokinase by the accumulation of mannose-6-phosphate, a balance can exist between the breakdown of ATP by hexokinase and the generation of ATP by the glycolytic metabolism of mannose. A similar balance between 6-phosphogluconate dehydrogenase and glucose-6-dehydrogenase enzyme activities affects viability in *Drosophila melanogaster* (Eanes 1984).

Exposure of barnacles to physiological stress may alter the ATP balance by causing a shift from aerobic to anaerobic metabolism. Less ATP would then be generated by metabolism of each mannose molecule. In *S. balanoides*, prolonged exposure to temperature and/or desiccation stress causes a fivefold decrease in oxygen consumption (Grainger & Newell 1965) and limits aerobic respiration (Barnes *et al.* 1963).

This study suggests that the combination of dietary mannose and temperature stress is the selective agent that structures patterns of *Mpi* genotype-frequency variation in nature. Under such environmental conditions, MPI activity may affect survivorship by altering the balance between ATP use and genesis. The significant genotypic differences in growth and survivorship indicate that *Mpi* genotypes may differ in their effective ability to process mannose-6-phosphate through glycolysis, perhaps due to underlying allelic differences in kinetic parameters. Without detailed functional studies, however, the hypothesis of selection at a locus in linkage disequilibrium with *Mpi* cannot be rejected (Clarke 1975). The link between dietary mannose and genotypic survivorship established here suggests that any selected linked locus would also need to be involved in the metabolism of mannose. Cis-acting regulatory elements that affect gene expression would seem to be logical candidates (Crawford & Powers 1992; Laurie *et al.* 1991).

There is mounting evidence that polymorphism at *Mpi* is directly and actively maintained by selection regimes that vary across habitats in the intertidal zone. Previous studies indicated viability selection on alternative homozygous genotypes in different environments (Schmidt & Rand 1999; Schmidt *et al.* 2000). The selective patterns observed in this experiment were identical to those observed in natural habitats characterized by high levels of thermal stress; however, there was no evidence of selection either against the *Mpi*-FF genotype or favouring the *Mpi*-SS genotype in these laboratory manipulations. Further examination of other aspects of fitness, such as genotype- and environment-specific fecundity, may elucidate other factors contributing to the maintenance of genetic polymorphism in this system.

Mpi is strongly polymorphic in barnacles and other crustaceans (Hedgecock *et al.* 1982) and selective patterns of genotype-frequency variation across intertidal habitats have been documented in the barnacle *Balanus glandula* (Hedgecock 1986) as well as nine species of amphipods (McDonald 1991; Siegismund 1985). Given the relationship between dietary mannose and organismal growth and survivorship established here, the *Mpi* locus may represent a general target of selection in a variety of marine taxa.

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