Adaptation at Specific Loci. VI. Divergence vs. Parallelism of Polymorphic Allozymes in Molecular Function and Fitness-Component Effects Among *Colias* Species (Lepidoptera, Pieridae)

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In lowland *Colias* butterflies, genotypes of the enzyme phosphoglucose isomerase (PGI) show major differences in molecular function, from which genotypic differences in organismal performance and fitness components in the wild are accurately predictable. The alpine species *Colias meadii* seems to share electromorph alleles with lowland congeners at PGI and phosphoglucomutase (PGM). However, high-resolution electrophoresis finds differences between PGI electromorphs of *meadii* and those of lowland taxa. Common *C. meadii* genotypes differ in thermal stability and are less thermally stable than similar electromorph genotypes in lowland *Colias eurytheme*. These *meadii* genotypes show heterozygote advantage in the kinetic parameters K_m and V_{max}/K_m (and differ sharply from genotypes of *C. eurytheme*). The thermally more stable homozygote is the kinetically less effective one, extending the tradeoff of kinetics vs. stability in PGI homozygotes, seen in lowland taxa, to *C. meadii*. Positive evidence is given for the absence of assortative mating and segregation distortion at both PGI and PGM. The functional differences among PGI genotypes explain previously observed heterozygote advantage in flight capacity and survivorship and correctly predict heterozygote advantage in male mating success, of *C. meadii*'s PGI genotypes. Though functional information is not yet available on *C. meadii* PGM variants, these also show heterozygote advantage in male mating success but do *not* interact with PGI.

Thus, differences in molecular function result in fitness component differences among PGI allozymes in alpine *Colias*, as well as in lowland ones. This is the more remarkable because the parallelism is *not* based on allelic identity. These results support expectations of evolutionary bioenergetics and emphasize the hazards of using ordinary electrophoresis to infer allozymes' identity among taxa. In alternative scenarios for *Colias*' PGI evolution—divergence from ancestral polymorphism, or independent origin—these results suggest major constraint, based in protein structure, on ability of PGI alleles to maximize fitness-related biochemical performance when homozygous.

Introduction

Darwin (1859) identified two major problems for evolutionary biology to solve: the origin and increase both of organisms' adaptation to their environments and of living diversity. Since then, insight into diversity has been gained, albeit controversially (e.g., Mayr and Ashlock 1991; Ridley 1986). In contrast, we still struggle with the "definition and recognition of adaptation" (Bock 1980). Comparative biochemistry and physiological ecology find taxonomic differences in adaptation—yet to explore these rigorously, other approaches are needed (Feder 1987). To sort out adaptations from constrained or neutral states (cf. Gould 1980), we can study any changes in fitness that are produced as natural genetic variants interact with ecological niche structures (Feder and Watt 1992). This approach began some time ago (e.g., Watt 1968; Koehn 1969; Day, Hillier, and Clarke 1974*a*, 1974*b*), but its use was impeded by extreme arguments arising from the "neutralist–selectionist debate" (Watt 1995). Joint molecular, organismal, and ecological studies now show that some natural variants are neutral while others undergo strong selection (e.g., Dean 1989; Powers et al. 1991; Clark and Koehn 1992; Karl and Avise 1992; Berry and Kreitman 1993; Begun and Aquadro 1994; Pogson and Zouros 1994; Watt 1994). Some molecular variants have long histories, e.g., vertebrate histocompatibility alleles (Nei and Hughes 1991; Takahata, Sata, and Klein 1992; Hedrick 1994) or plant self-incompatibility alleles (Ioerger, Clark, and Kao 1990). Study of present adaptedness and phyletic history of natural variants among differently related taxa can begin to answer important questions: How do mechanisms of adaptation and constraint change with ecological shifts and/or phyletic diversification (cf. Jermann et al. 1995)? How general or repeatable is the evolution of such mechanisms (cf. Harvey and Pagel 1991)?

We have studied molecular adaptation in carbohydrate-processing allozymes in the closely related American lowland butterflies, *Colias eurytheme* Boisduval and *Colias philodice eriphyle* Edwards. Glycolysis is an important context for this: it supplies "fuel" to flight, and all adult *Colias* fitness components depend directly on flight (Watt 1977, 1992). In glycolysis, phosphoglucose isomerase (PGI) interconverts glucose- and fructose-6-phosphates (G6P, F6P). In lowland *Colias*, there are major differences in kinetics and thermal stability among PGIs of common allozyme genotypes, with predictable effects on in vivo metabolic performance (Watt 1977, 1983; Watt and Boggs 1987). Especially in homozygotes, there is a strong negative correlation or

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"tradeoff" between kinetic effectiveness and thermal stability; such a tradeoff was predicted from general protein structure principles by Hochachka and Somero (1973, chapt. 7). These functional differences correctly predict genotypic differences in insect flight capacity in the wild (Watt, Cassin, and Swan 1983). In turn, these flight differences accurately predict genotype-specific survival, male mating success, and female fecundity (Watt 1983, 1992; Watt, Carter, and Blower 1985; Watt, Carter, and Donohue 1986). Variants of phosphoglucomutase (PGM) and glucose-6-phosphate dehydrogenase (G6PD), reactions leading from G6P to other metabolic pathways, show genotypic differences in male mating success (but in neither flight capacity nor survivorship), which are statistically independent of those at PGI (Carter and Watt 1988).

Here, we ask how these molecular adaptive mechanisms vary with species and/or their niche structures. We begin with *Colias meadii* Edwards, found in alpine grasslands of the American Rocky Mountains. We ask if PGI alleles of *C. meadii* and lowland taxa, seemingly the same in ordinary electrophoresis, are really so at higher resolution. *Colias meadii* PGI genotypes are known to differ in flight activity and survivorship (Watt 1977, 1983); we ask if differences in function among these genotypes can explain their flight and survival differences. We ask if the tradeoff of kinetics vs. thermal stability applies to *C. meadii* PGI. Finally, we study male mating success, predicting that *C. meadii* PGI allozymes' functional differences cause their genotypes to differ in this additional major fitness component, and testing that prediction.

Materials and Methods

Experimental Insects

Colias meadii were sampled at Cumberland and Cottonwood Passes, Gunnison County, and at Mesa Seco, Hinsdale County, both in Colorado. Population structures are known at Cumberland Pass and Mesa Seco (Watt et al. 1977). *Colias meadii* were reared in the laboratory on clover (*Trifolium*) at 27–30°C and 16 h light : 8 h dark photoperiod.

Electrophoresis

Polyacrylamide gel electrophoresis for allozyme genotyping was done following Carter and Watt (1988). For higher resolution, Ferguson plots (Rodbard and Chrambach 1971) were made. Individual insects' extracts were electrophoresed repetitively at four gel porosities (7–10% acrylamide), with ferritin and myoglobin, and the extreme-mobility *Colias* PGI genotype 5/5, as internal standards. Band positions were measured with a Gilford scanner (Watt 1977); standards were used to normalize mobilities of tested PGI bands, correcting for gel–gel variation (Watt 1977). Log-transformed electrophoretic mobilities were regressed against porosity, estimating the logs of enzymes' free mobilities, ln M_o, as Y-intercepts and retardation coefficients, K_r, as slopes. As a control against posttranslational modification, only heterozygotes were studied in Ferguson plots. If both homodimers of a heterozygote show parallel deviation in K_r or M_o from normal values of their electromorph alleles in such plots, the data may reflect a posttranslational effect; one such case was found in *C. p. eriphyle* (Watt 1977).

Enzyme Purification

These steps were used to purify PGI: (1) 1 h centrifugation of electrophoresis extracts, pooled by genotype, at 40,000 × *g* to remove cell debris and glycogen; (2) fractional precipitation of PGI between 1.5 M and 2.5 M ammonium sulfate at pH 7.5—yield 95% with 4–5-fold purification; (3) molecular sieving through Pharmacia Sephacryl SR-300 in 0.05 M potassium phosphate, pH 7.5—yield 90% with 10-fold further purification; (4) ion-exchange filtration, after desalting, through BioRad BioGel-DEAE in 0.01 M Tris buffer at pH 7.4, whence PGI emerges at the void volume while most other material is adsorbed—yield 70% with 10–12-fold purification, to a total of 400–500× purification. PGI thus prepared is not yet 100% pure (Villa and Watt, unpublished) but does not change in kinetics or stability after step 3 and is stable for years if stored at 4°C.

PGI Enzyme Assay and Kinetics

Routine PGI assays followed Watt (1977, 1983): initial velocity in the gluconeogenic direction (F6P → G6P) was measured by coupling to excess G6PD and measuring reduction of NAD^+ or $NADP^+$ (depending on requirements of the G6PD used) at 340 nm and 30°C, pH 8, in thermostatted Gilford spectrophotometers.

Kinetic parameters of interest are the Michaelis constant K_m (equal to substrate concentration giving half-maximal velocity, hence a relative measure of substrate affinity), V_{max} as maximum saturated activity/mg protein, and the "pseudo-first-order rate constant" V_{max}/K_m, which determines individual enzyme steps' contribution to control of transient metabolic response (and steady-state flux) to biological demand (Watt 1983, 1992, references therein). The glycolytic reaction direction (G6P → F6P) is of primary importance in adult flight muscle. However, the Haldane equation

$$K_{eq} = \frac{(V_{max}/K_m)_f}{(V_{max}/K_m)_b}$$

(K_{eq} = equilibrium constant, f = forward, b = back) ensures that V_{max}/K_m differences in the glycolytic direction (f) are exactly proportional to those in the gluconeogenic direction (b; Watt 1983). Thus, for convenience, we measured kinetics in the latter direction. K_m was measured with [F6P]/v vs. [F6P] plots, at four concentrations bracketing the K_m range expected from initial data (10, 50, 250, and 1,250 μM), with assays in triplicate at each concentration. Plots were calculated with a repeated-Y-per-X regression design that allows sensitive test of plot linearity (Sokal and Rohlf 1995), and were replicated 5–10 times per genotype. Maximum specific activity, V_{max}/mg protein, was measured in fresh homogenates from newly emerged adults, "scrubbed" of low-molecular-weight metabolites by molecular sieving through BioRad Biogel P6. Knowing K_m for each genotype, we

calculated substrate concentration ([F6P] = $19K_m$) producing v = 95% V_{max} for each genotype, assayed each individual homogenate in triplicate at that concentration, and corrected by 1/0.95 to obtain V_{max}. Protein was assayed with the ultraviolet protein assay of Waddell (1957); details follow Watt (1977) with minor modifications. The enzyme assays for K_m and V_{max} measurements were all carried out at 30°C in 0.05 M imidazole buffer plus 0.01 M KCl and $MgCl_2$, titrated to pH 6.92 at 23°C, to match the in vivo pH curve of most ectotherm animal cytosols, which follows the ionization of the histidine imidazole ring (Yancey and Somero 1978).

PGI Thermal Stability Measurement

The kinetics of protein denaturation can be very complex (e.g., Laidler and Bunting 1973), so thermal stability study over short time intervals may be most simply informative. Thermal stability of PGI was measured in 0.05 M imidazole buffer titrated to pH 6.92 at 23°C, thus also on the in vivo pH-vs.-temperature curve of Yancey and Somero (1978). Denaturation temperatures between 47 and 51°C, with spacing chosen to maximize resolution, were used. In the pH range corresponding to this temperature range, *Colias* PGI's stability changes only slowly (unpublished) so effects seen *are* mainly due to temperature rather than pH. Allozyme preparations were diluted 1:10 into the imidazole buffer at the experimental temperature, duplicate aliquots were drawn at once for 0-min assay, and the dilution was incubated for 20 min in a stirred water bath at experimental temperature ±0.2°C; duplicate aliquots were then taken to measure activity retained. The difference between rates at 0 min and at 20 min incubation was the loss of activity, "v," for analysis (see below).

The Arrhenius equation for thermal dependence of a process' rate is

$$k = Ae^{-(E_{act}/RT)}$$

where k is the rate constant, E_{act} is the process' free energy of activation, R is the gas constant, T is temperature (°K), and the constant A expresses other information from which, together with E_{act}, the enthalpy and entropy of activation may be calculated (e.g., Laidler and Bunting 1973). If we plot the logarithm of k against $1/T$, the plot's slope is E_{act}/R and its intercept gives the constant A. Our enzyme is not 100% pure, so (saturated) assay rates "v" are proportional to, but not equal to, molar rate constants k, whether for catalysis (k_{cat}) or for denaturation. In the latter case, of interest here, "v" is the loss of activity over 20 min (above). For each enzyme genotype, "v" contains a preparation-specific unknown value for enzyme concentration [E], multiplied by k:

$$v = k[E] = A[E]e^{-(E_{act}/RT)}$$

The logarithmic transformation $\ln v = \ln (k[E]) = \ln k + \ln [E] = \ln A + \ln [E] - E_{act}/RT$ removes [E] from the slope of an Arrhenius plot, though not from the intercept ($=\ln A + \ln [E]$). This does allow us to calculate E_{act} for PGI denaturation from genotype- and preparation-specific plots of ln (% loss of activity per 20 min) vs. $1/T$.

Male Mating Success Measurement

Our protocol for studying genotype-specific male mating success in the wild (Watt, Carter, and Blower 1985; Watt, Carter, and Donohue 1986) depends on knowing that all eggs laid by a female at one time have only a single father. Obviously this is true for singly mated females. In the laboratory (Boggs and Watt 1981) and in the field (Carter and Watt 1988), when a lowland *Colias* female mates more than once, the last male mating her fathers all her later eggs; i.e., sperm precedence is complete. This can now be verified for *C. meadii* as well. Multiply mated females are identified by dissecting them to count spermatophores before they are genotyped (Watt, Carter, and Donohue 1986). Following Carter and Watt (1988), we calculated the fraction of our 51 broods, obtained from multiply-mated female *C. meadii,* which should have shown more than two paternal alleles at PGI or PGM if uniform sperm mixing occurred (fewer if mixing were incomplete). This fraction would be 10–20%, calculated using PGI genotype frequencies, or 33–49%, calculated with PGM frequencies. We found no such evidence for sperm mixing at either gene in any of the 51 multiple-mating cases, so within that resolution, eggs laid at one time by a singly *or* multiply mated *C. meadii* female have only one father.

Given this, wild samples of adults were taken during peak daily flight; males were genotyped; eggs were obtained from females, which were then dissected for spermatophore counting and genotyping; enough larvae from each female were then genotyped to determine their fathers' genotypes with ≥99% confidence. For a homozygous female, 7 progeny showing only one paternal allele support paternal homozygosity at >99% confidence, while for a heterozygous female, 16 larvae may be required, depending on probability of ratios observed (Watt, Carter, and Blower 1985). In practice, larvae from each brood were electrophoresed in groups of four, until paternal genotype for each gene was resolved. This yields sample genotype arrays for viable vs. successfully mating males, hence estimates of genotype-specific male mating success.

Statistical Analysis

Statistical testing was done with a standard computer package (SYSTAT 5.0), or with computer programs written to execute specialized routines (Goldstein 1964; Sokal and Rohlf 1995). Common abbreviations: ANOVA, analysis of variance; F, variance ratio test statistic; G, log likelihood ratio statistic; P, probability of obtaining a result by chance deviation; x^*, exact binomial test statistic for difference of proportions.

Results

Description of Polymorphisms and Identity of Electromorphs

Table 1 exemplifies allele and genotype frequencies for *C. meadii* PGI, PGM, and G6PD (cf. Watt 1977, 1983, and Carter and Watt 1988 for comparable data on lowland taxa). Johnson (1975) reported high heterozygosity for G6PD in this *C. meadii* population, but we

Table 1
Typical Genetic Statistics for *C. meadii* Allozyme Polymorphisms

	Alleles and Frequencies						
Locus	1	2	3	4	5	H	ΔH
PGI	0.013	0.554	0.407	0.026		0.513	−0.013
PGM	0.146	0.010	0.364	0.454	0.026	0.642	+0.003
G6PD ...	0.993	0.007				0.013	+0.0001

	Genotypes and Frequencies														
	1/1	1/2	1/3	1/4	1/5	2/2	2/3	2/4	2/5	3/3	3/4	3/5	4/4	4/5	5/5
PGI ...	0.000	0.019	0.006	0.000		0.314	0.436	0.026		0.173	0.026		0.000		
PGM ..	0.013	0.007	0.079	0.172	0.007	0.000	0.000	0.013	0.000	0.146	0.331	0.026	0.192	0.007	0.007
G6PD ..	0.987	0.013				0.000									

Note.—Sample of 156 young insects (mean wear rating 2.9 ± 0.6; cf. Watt et al. 1977) of both sexes taken at midday (peak flight period) at the Mesa Seco, Hinsdale Col., Colorado, elevation 3,535 m, 5 August 1988. Electromorph allele numbers are simply labels (cf. Carter and Watt 1988) and carry no implications of cross-locus correspondence whatsoever. H = heterozygosity. ΔH = deviation of heterozygosity from Hardy–Weinberg expectations—in this sample, quite insignificant for all three loci. Males and females differ in allele or genotype frequencies by at most 2–5%.

find one common allele, similar to the 1 electromorph of the lowland species complex, while an allele like the 2 electromorph occurs at very low frequency. Carter and Watt (1988) found nonheritable multiple banding for G6PD when $NADP^+$ was absent from the upper electrode buffer in electrophoresis; this may explain Johnson's G6PD results. (Johnson's heterozygosities for PGM do resemble ours.) Because G6PD is so nearly monomorphic in *C. meadii,* we do not study it further here. For convenience, we number electromorph alleles of PGI or PGM in order of increasing mobility (Carter and Watt 1988), without implying molecular identity among taxa.

Colias meadii seems to share PGI and PGM alleles with lowland taxa in normal-resolution acrylamide gels (Watt 1977; Carter and Watt 1988). We tested this further with Ferguson plots, as above. Table 2 shows that PGI electromorphs of *C. meadii* differ both in retardation coefficient, K_r, and log free mobility, ln M_o, from superficially similar electromorphs of *C. p. eriphyle.* This contrasts to earlier findings that PGI alleles of *C. eurytheme* and *C. p. eriphyle* do *not* differ in K_r or ln M_o (Watt 1977).

PGI Thermal Stability Among Genotypes and Taxa

We examined PGI genotypic thermal stability in *C. meadii* and in *C. eurytheme* for comparison. Figure 1 presents plots of PGI thermal stability for the most common *C. meadii* genotypes, 2/2, 2/3, and 3/3, and, for comparison, similar electromorph genotypes plus the very stable 4/4 genotype from *C. eurytheme* (the corresponding electromorph genotype is extremely rare in *C. meadii*). Table 3 gives energies of activation for PGI protein denaturation, calculated from these data, including a significance test for each plot and confidence limits on each slope ($=E_{act}/R$).

Major genotypic differences in thermal stability are found among *C. meadii* PGI allozymes. Values of E_{act} for denaturation span most of the range of values known for different proteins (e.g., Laidler and Bunting 1973); *C. meadii*'s 3/3 is particularly heat labile. At this pH, *C. eurytheme* 2/3 shows a slight heterozygote advantage in

Table 2
Ferguson-Plot Analysis of Identity of Electromorph Alleles Across Species

Electromorph Allele	Species	n	K_r Mean ± SD	K_r ANOVA Significance	ln M_o Mean ± SD	ln M_o ANOVA Significance
2	*eriphyle*	4	0.083 ± 0.010	$F_{1,9} = 7.9$	−1.830 ± 0.150	$F_{1,9} = 7.3$
	meadii	7	0.128 ± 0.031	$P = 0.020$	−2.204 ± 0.248	$P = 0.024$
3	*eriphyle*	9	0.105 ± 0.011	$F_{1,17} = 7.1$	−1.537 ± 0.122	$F_{1,17} = 5.5$
	meadii	10	0.124 ± 0.019	$P = 0.016$	−1.697 ± 0.169	$P = 0.032$
4	*eriphyle*	8	0.110 ± 0.004	$F_{1,8} = 29.2$	−1.298 ± 0.051	$F_{1,8} = 10.5$
	meadii	2	0.126	$P = 0.0006$	−1.434	$P = 0.012$

Note.—Insects electrophoresed at four acrylamide gel percentages: 7.02, 8.03, 9.03, 10.04; data are for homodimers of heterozygotes only, as a control against nonspecific posttranslational modification (cf. Watt 1977). Allelic homodimer mobilities were measured relative to ferritin and PGI (5/5 homozygote) internal standards, then log-transformed, and regressed on acrylamide percentage; slope of the plot is the retardation coefficient K_r, while the y-intercept is $\ln(M_o)$ = ln(free mobility) (Rodbard and Chrambach 1971). SD = standard deviation. ANOVA shows that what seem, in low porosity gels, to be "the same" electromorphs are, in fact, different.

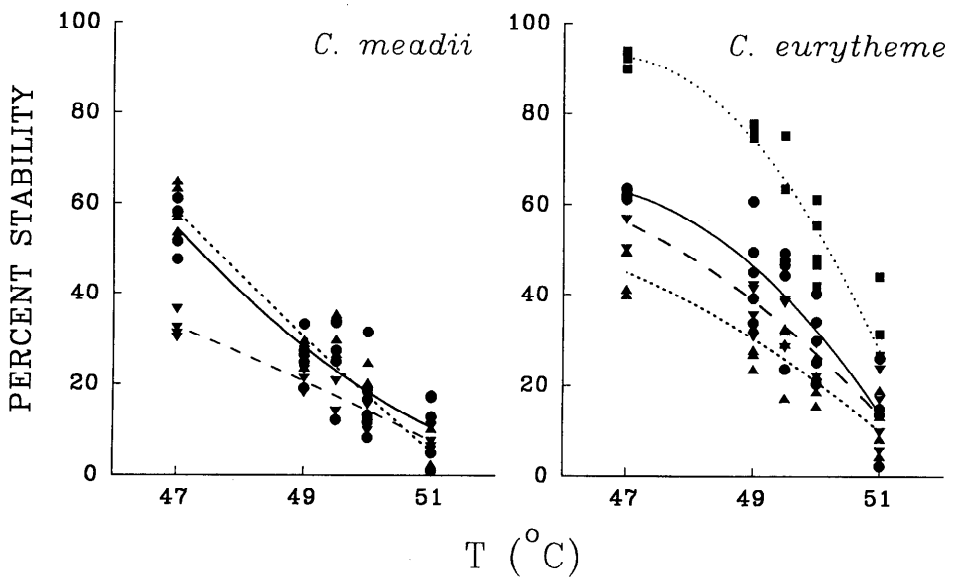

FIG. 1.—Thermal stability of PGI allozyme genotypes of *Colias meadii* and of *C. eurytheme* for comparison. Percent stability (ordinate) is the fraction of starting activity remaining after 20 min incubation at experimental temperature (abscissa). Lines fitted by least squares. Data for similar electromorphs (*not* identical, see table 2) plotted with the same symbols and lines between taxa: 2/2, inverted triangles and long dashes; 2/3, circles and solid lines; 3/3, upright triangles and short dashes; 4/4, squares and dots. See table 3 for energies of activation for denaturation and statistics, based on these data. See text for experimental design and interpretation.

stability, whereas at higher pH the order is 3/3 > 2/3 >> 2/2 (Watt 1977). Homozygote stabilities are ordered in reverse among electromorphs between the species, and *C. meadii*'s genotypes are generally less stable than those of *eurytheme*. This result emphasizes (as do kinetics comparisons below) that electromorph similarity across taxa does *not* imply functional similarity. It has environmental implications as well (see Discussion).

PGI Kinetics Differences Among Genotypes and Taxa

Figure 2 presents K_m values for F6P for the three common *C. meadii* PGI genotypes and the four PGI genotypes of *C. eurytheme* compared above for thermal stability. Table 4 presents V_{max}/mg protein data and V_{max}/K_m ratio values for *C. meadii*. The *C. meadii* PGI genotypes' K_m's are numerically ordered 2/3 < 3/3 < 2/2, thus showing highly significant heterozygote advantage in relative substrate affinity, as verified by a post hoc contrast test (fig. 2 caption). Homozygote K_m disadvantage for *C. meadii* is quite asymmetric. Specific activity differences among genotypes are small, showing dominance of the two alleles but are not yet significant. (The genotypic order of mean specific activities has been consistent from the first, so an even larger sample may confirm its reality.) Resulting V_{max}/K_m ratios also display strong heterozygote advantage.

Table 3
Energy-of-Activation for Thermal Denaturation of *Colias'* PGI Genotypes

Electromorph Genotype	Species	Slope ± 95% c.l.	Significance tests: Regression	Significance tests: Deviation	E_{act}(kJ/mol ± 95% c.l.)
4/4	*eurytheme*	−58,171 ± 7,287	$F_{1,3} = 245.3$ $P = 0.0005$	$F_{3,14} = 1.60$ $P = 0.23$	484 ± 61
3/3	*eurytheme*	−17,589 ± 1,846	$F_{1,3} = 349.3$ $P = 0.0003$	$F_{3,16} = 0.33$ $P = 0.81$	146 ± 15
	meadii	−8,184 ± 1,083	$F_{1,3} = 219.7$ $P = 0.0006$	$F_{3,15} = 0.78$ $P = 0.53$	68 ± 9
2/3	*eurytheme*	−21,392 ± 3,704	$F_{1,3} = 128.4$ $P = 0.001$	$F_{3,19} = 0.67$ $P = 0.58$	178 ± 31
	meadii	−17,782 ± 5,086	$F_{1,3} = 47.1$ $P = 0.005$	$F_{3,21} = 2.47$ $P = 0.09$	148 ± 42
2/2	*eurytheme*	−12,761 ± 1,244	$F_{1,3} = 405.6$ $P = 0.0003$	$F_{3,18} = 0.26$ $P = 0.85$	106 ± 10
	meadii	−21,712 ± 3,857	$F_{1,3} = 122.0$ $P = 0.001$	$F_{3,20} = 2.45$ $P = 0.09$	181 ± 32

NOTE.—Data were first converted to fraction of activity *lost* in 20 min at the several experimental temperatures. The natural log of this was then regressed against $1/T$ in °K, giving a line whose slope is E_{act}/R, where R is the gas constant and E_{act} is the energy of activation for thermal denaturation (see text). 95% c.l. = 95% confidence limits. The regression design used allows for test of each line's deviation from linearity (Deviation) as well as the test for significance of the slope itself (Regression) (Sokal and Rohlf 1995).

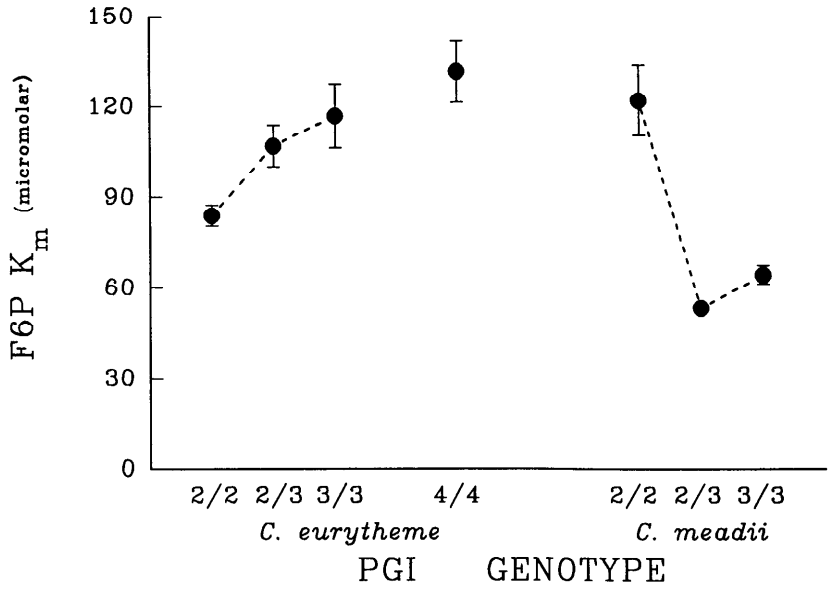

FIG. 2.—Michaelis constants, K_m, for F6P for PGI of *Colias meadii* and of similar electromorphs of the lowland *C. eurytheme* for comparison. Error bars are standard errors of means; for *C. meadii* genotype 2/3, they are present but contained within the primary symbol! Nested analysis of variance shows that both species and genotypes are extremely significantly different in K_m: for species, $F_{1,32} = 36.9$, $P = 8.7 \times 10^{-7}$; for genotypes nested under species, $F_{5,32} = 18.8$, $P < 1 \times 10^{-8}$. In ANOVA of *C. meadii* alone, a specific post hoc contrast testing the hypothesis 2/3 K_m < 3/3 K_m < 2/2 K_m is also extremely significant: $F_{1,19} = 78.9$, $P = 3 \times 10^{-8}$! See also table 4.

The kinetically more effective homozygote 3/3 is much less thermally stable than the kinetically inferior 2/2. Thus the Hochachka–Somero (1973) tradeoff of thermal stability vs. kinetics occurs in *C. meadii* PGI homozygotes as well as those of lowland taxa (Watt 1977, 1992) but with different electromorphs playing the different roles. Because 3/3 is much less stable than 2/3, its V_{max}/K_m disadvantage is predicted to increase with age in the wild as the insects accumulate thermal stress exposure.

Test for Assortative Mating and Segregation Distortion Among Allozyme Genotypes

A major purpose here is to test de novo predictions of *Colias* PGI genotypic male mating success differences that arise from the molecular properties of the allozymes. When studying male mating success, we must test for assortative mating, as if this occurred, it could seriously bias our results. As table 5 shows, we tested, for PGI and for PGM, the fractions of heterozygous males mating all females and of heterozygous males mating heterozygous females. These fractions never differ significantly, for any sample or for all samples pooled at either gene, nor are there consistent trends. This is strong evidence against assortative mating among genotypes at either gene.

Further, among the larval broods of *C. meadii* reared for these paternity determinations, there are some extreme segregation ratios. Because some possible interpretations of this could indicate biases in our mating success design, we inquired further into these data. We consider only crosses in which the parents carry at least three alleles, e.g., A/B × A/C, or else only one parent is a heterozygote. In A/B × A/B progenies, one cannot tell which parent contributed which allele to an A/B offspring. At each locus there were a large number of x^* tests, so we had to adjust the minimally significant probability level α. We did so by the Dunn–Sidák method (Sokal and Rohlf 1995): the adjusted significance threshold $\alpha' = 1 - (1 - \alpha)^{1/k}$, where k = the number of tests per question and gene locus, and $\alpha = 0.05$ as

Table 4
Kinetic Parameters of *C. meadii* PGI Genotypes

GENOTYPE	2/2	2/3	3/3
K_m μM	122 ± 12	53 ± 1.9	64 ± 3.0
n	5	7	10
V_{max}/mg protein, EC units/mg	0.458 ± 0.052	0.453 ± 0.028	0.421 ± 0.022
n	8	10	8
V_{max}/K_m EC units/mg/μM	3.8×10^{-3}	8.6×10^{-3}	6.6×10^{-3}

NOTE.—F6P-K_m values taken from figure 2. These and V_{max}/mg protein data are means ± standard errors of means. K_m differences, determined on highly purified preparations (see text) are highly significant as reported in caption to figure 2. V_{max}/mg protein values, determined on fresh homogenates scrubbed of small metabolites (see text), are given in the EC (=Enzyme Commission) unit, defined as 1 μmol substrate converted per minute; they are not significantly different by ANOVA: $F_{2,23} = 0.31$, $P = 0.74$. Heterozygote advantage in V_{max}/K_m is due primarily to the K_m differences.

Table 5
Test for Assortative Mating of *C. meadii* Allozyme Genotypes

Sample	Heterozygote Males Mating Females for Each Locus			
	PGI		PGM	
	Fraction	%	Fraction	%
Cumberland Pass 1985				
All females	34/47	0.723	36/44	0.818
Heterozygote females	26/36	0.722	25/31	0.806
	$x^* = 0.01$, $P > 0.9$		$x^* = 0.13$, $P = 0.9$	
Cottonwood Pass 1986				
All females	34/50	0.680	36/48	0.750
Heterozygote females	14/22	0.636	21/30	0.700
	$x^* = 0.36$, $P = 0.72$		$x^* = 0.48$, $P = 0.64$	
Mesa Seco 1988				
All females	43/70	0.614	57/69	0.826
Heterozygote females	25/38	0.658	37/45	0.822
	$x^* = -0.45$, $P = 0.66$		$x^* = 0.05$, $P > 0.9$	
Cottonwood Pass 1989				
All females	21/42	0.500	34/46	0.739
Heterozygote females	10/14	0.714	24/32	0.750
	$x^* = -1.40$, $P = 0.08$		$x^* = -0.06$, $P > 0.9$	
Cottonwood Pass 1991				
All females	40/62	0.645	55/63	0.902
Heterozygote females	22/33	0.667	43/45	0.956
	$x^* = -0.21$, $P = 0.82$		$x^* = -1.04$, $P = 0.30$	
Total				
All females	172/271	0.635	214/266	0.805
Heterozygote females	97/143	0.678	150/183	0.820
	$x^* = -0.89$, $P = 0.38$		$x^* = -0.40$, $P = 0.60$	

Note.—Heterozygosity of males successfully mating all females, vs. those mating heterozygote females only. x^* = Goldstein's (1964) exact binomial test statistic for difference of proportions.

Table 6
Expected and Observed Segregation Ratios for 16-Larva Allozyme Broods of *C. meadii*

Progeny ratios	PGI			PGM		
	Obs	Exp		Obs	Exp	
15:1, 1:15	1	0.02	0.72	0	0.01	1.69
14:2, 2:14	0	0.12		1	0.08	
13:3, 3:13	0	0.58		1	0.38	
12:4, 4:12	2	1.89		1	1.22	
11:5, 5:11	4	4.53		5	2.93	
10:6, 6:10	10	8.31		6	5.37	
9:7, 7:9	10	11.87		5	7.68	
8:8	7	6.68		3	4.32	
	$G_{(5)} = 0.82$, $P > 0.9$			$G_{(4)} = 3.64$, $0.3 < P < 0.5$		

Note.—Bracketed classes pooled for G goodness-of-fit test between observed and expected values. For discussion of experimental design, see the text.

usual. These data are bulky, so we summarize the analysis; details are available from the corresponding author.

First, did overall segregation ratios favor particular alleles at either gene? Segregation distortion, as found in *Drosophila,* e.g., Temin and Marthas (1984), at either gene could change allele, and hence genotype, frequency dynamics in the wild. The adjusted significance thresholds were: PGI, $\alpha' = 0.0102$; PGM, $\alpha' = 0.0051$. At these significance levels, for PGI, the 1 allele was disfavored among progeny of 1/2 heterozygotes, whereas at PGM, 2-alleles were disfavored among progeny of 2/3 and 2/4 heterozygotes.

Second, was there bias for or against heterozygotes among progeny? A bias favoring heterozygosity could represent "associative overdominance," with deleterious recessives closely linked to allozyme alleles so that homozygotes were disfavored (e.g., Zouros 1987). There were no such biases favoring heterozygotes, significant at α' for PGI, $P = 0.0102$, or for PGM, $P = 0.009$. In fact, *homozygotes* were favored (though not significantly) among progeny of PGI 1/2 and PGM 2/3 and 2/4. The apparent transmission disadvantages of PGI allele 1 and PGM allele 2 cannot reflect linkage to deleterious recessives, because such linkage would *dis*favor homozygotes and favor heterozygotes.

Third, are the segregation ratios any more extreme than one would expect? In our paternity protocol, larval broods $4 < n \leq 8$ in number only arise when the first four larvae from a homozygous female segregate as 0:4 or 4:0, so four more larvae are run to resolve the paternity. This biases eight-larva broods toward extreme ratios. To eliminate this bias, we can study segregations at PGI or PGM in 16-larva progenies. Such large progenies are not scored to test paternity for a female homozygote, because seven (in practice, eight, as above) larvae pass the 99% criterion for paternity in that case. They arise while testing paternity for females heterozygous at a different gene, so that up to 16 larvae may be needed for 99% confidence of paternity if the father is homozygous (Watt, Carter, and Blower 1985). Table 6 shows expected and observed segregation data among 16-larva progenies; they agree closely for both PGI and PGM. When design-induced bias is removed, there is no excess of extreme segregations; this is strong evidence against segregation distortion. The occasional bias against larval heterozygotes of the uncommon PGI 1- and PGM 2-alleles may reflect viability disadvantage in our warm rearing conditions.

Male Mating Success Differences Among Allozyme Genotypes

Colias flight performance depends directly on speed of glycolytic transient response to flight-muscle ATP depletion. Therefore, PGI is heavily selected to maintain maximum values of V_{max}/K_m, with respect both to kinetics and to the stability of PGI once synthesized. In turn, adult fitness components depend on flight performance (Watt 1968, 1977, 1983, 1992). Therefore, the combination of genotype-specific kinetics and stability values found for *C. meadii* explains 2/3's advantages in flight capacity and survivorship, previously observed (Watt 1977, 1983). Further, because flight capacity is of primary importance to *Colias* males' ability to find fe-

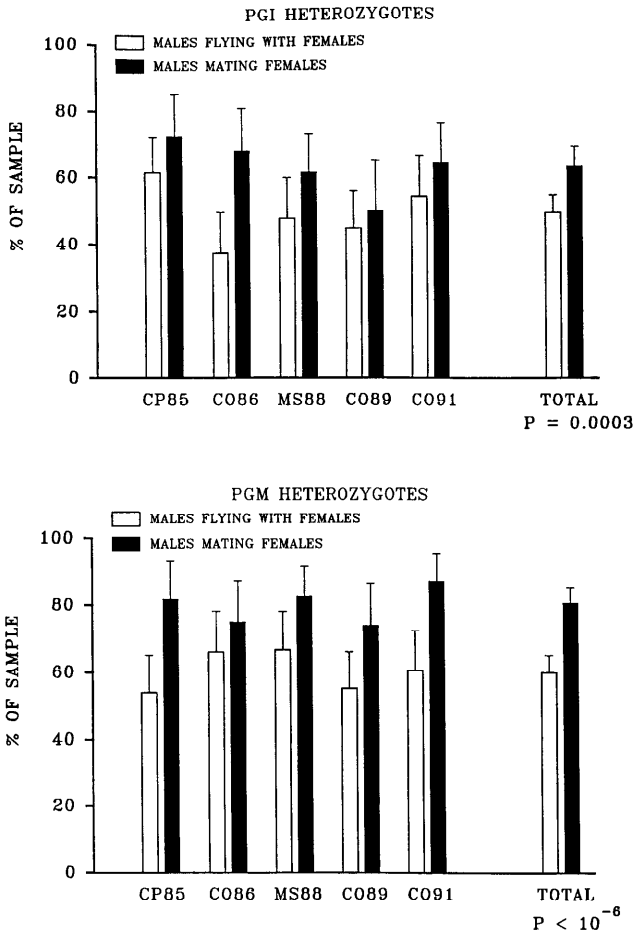

FIG. 3.—Fractions of heterozygotes, for PGI and PGM loci, in samples of male *C. meadii* flying with females (open bars), vs. those successfully mating females (filled bars), in five separate samples and pooled totals. Error bars are 95% confidence limits on percentages (Goldstein 1964). Sample label abbreviations: digits = year of sample, e.g., "86" = 1986; CP = Cumberland Pass, Gunnison County, Colorado; CO = Cottonwood Pass, Gunnison County, Colorado; MS = Mesa Seco, Hinsdale County, Colorado; Total = five samples pooled. See table 7 for detailed statistical analysis; see text for further interpretation.

males and court them effectively (Watt 1968; Watt, Carter, and Blower 1985), the flight differences predict genotypic male mating success differences in *C. meadii* PGI. (By analogy with the lowland taxa, we also expect male mating success differences at PGM in *C. meadii.*) Results either of "no genotypic differences" or "opposite differences" would equally violate the predictions, so we maximize statistical power by using one-tailed tests. Otherwise we would be biased toward the Type II error (false acceptance of the null hypothesis).

Figure 3 and table 7 present male mating success data for PGI and PGM, which strongly support our predictions. For both genes, heterozygosity of the mating-males sample is always greater than that of the corresponding flying-males sample, as in lowland *Colias.* Because of logistically imposed limits on sample size, only two of the five PGI samples and four of the five PGM samples are significant by binomial test when standing alone. However, a one-tailed Sign Test of the mating success difference is significant for each gene ($P = 0.5^5 = 0.031$), and the pooled data are significant by binomial test beyond the $P = 0.001$ level for both genes. (Also, for both genes, mating-males samples always show heterozygote excess over Hardy–Weinberg expectations, whereas flying-males samples vary around Hardy–Weinberg agreement.)

Figure 4 gives genotype-specific analysis of the pooled sample data, comparing males mating females vs. males flying with females for both PGI and PGM. At both genes, observed heterozygote advantage in male mating success derives from the common heterozygote(s), as specifically predicted for PGI. Individual samples also show this pattern, though with variation as expected from their lesser sample sizes. In individual samples or the pooled data alike, there are no deviations from random association of genotypes at the two loci; that is, there is no epistasis for male mating success between PGI and PGM.

Discussion

Implications of Functional and Fitness Component Differences

The maintenance of PGI polymorphism under functionally predictable natural selection, in parallel among *Colias* taxa *but involving different alleles and genotypes,* is quite remarkable. As part of this, the recurrence of the "Hochachka–Somero tradeoff" of kinetics vs. stability in *C. meadii* PGI suggests that PGI structure may be constrained to this functional compromise through the whole genus. This is testable by further comparative study (cf. below).

Genotypic male mating success differences at *C. meadii*'s PGI are congruent with observed differences in survivorship among the same genotypes, as expected from their common origin in genotype-specific flight capacity (above; Watt 1983). Thus, as in the lowland species, we see so far no antagonism of fitness components arising from different life-history features, though such tradeoffs are expected by some theorists.

Functional study of the rarer *C. meadii* PGI genotypes will help us to understand fully the differences between selection operating on *C. meadii* and that on the lowland taxa. *Colias meadii* appears to occupy a thermal niche, which, compared to lowland taxa, is narrower and colder on average, though it does include recurrent overheating (Kingsolver and Watt 1983, 1984). Two of *C. meadii*'s three most common PGI genotypes are more thermally labile than their lowland electromorph counterparts, and all three are more labile than other common lowland genotypes. Thus, *C. meadii* may be even more vulnerable to possible global warming than its lowland relatives (cf. Watt 1992).

The magnitude of *C. meadii*'s PGI allozyme functional differences spans a somewhat smaller range, 2.5-fold, than that seen in lowland taxa, 4-fold (Watt 1977, 1983), and in turn the predicted and observed differences in magnitude of male mating success are smaller among the *C. meadii* genotypes, though robust in their occurrence as shown above. This agrees with the simple

Table 7
Male Mating Success vs. Allozyme Genotype in *C. meadii*

	PGI				PGM			
	Flying		Mating		Flying		Mating	
SAMPLE	H-W Match	*H*	H-W Match	*H*	H-W Match	*H*	H-W Match	*H*
Cumberland Pass 1985	−0.053	49/80	+0.140	34/47	−0.063	41/76	+0.101	36/44
		0.613		0.723		0.539		0.818
		$x^* = 1.27$, $P = 0.10$				$x^* = 3.07$, $P = 0.001$		
Cottonwood Pass 1986	−0.047	22/59	+0.140	34/50	−0.032	39/59	+0.118	36/48
		0.373		0.680		0.661		0.750
		$x^* = 3.20$, $P = 0.001$				$x^* = 1.00$, $P = 0.17$		
Mesa Seco 1988	−0.047	32/67	+0.091	43/70	+0.064	44/66	+0.153	57/69
		0.478		0.614		0.667		0.826
		$x^* = 1.61$, $P = 0.05$				$x^* = 2.13$, $P = 0.02$		
Cottonwood Pass 1989	−0.019	35/78	+0.075	21/42	−0.036	43/78	+0.040	34/46
		0.449		0.500		0.551		0.739
		$x^* = 0.54$, $P = 0.29$				$x^* = 2.08$, $P = 0.02$		
Cottonwood Pass 1991	+0.076	36/66	+0.143	40/62	−0.071	40/66	+0.143	55/63
		0.545		0.645		0.606		0.873
		$x^* = 1.15$, $P = 0.13$				$x^* = 3.44$, $P = 0.0002$		
Total		174/350		172/271		207/345		218/270
		0.497		0.635		0.600		0.807
		$x^* = 3.42$, $P = 0.0003$				$x^* = 5.52$, $P = 10^{-6}$		

NOTE.—Flying = sample of males flying with females, available as mates; Mating = sample of males actually succeeding in mating the females sampled. H-W match = excess (+) or deficiency (−) of heterozygote frequency compared to Hardy–Weinberg distribution for that sample and locus. *H* = heterozygotes/total genotypes for that sample and locus. x^* = Goldstein's (1964) exact binomial test for difference of percentages/proportions, applied to comparison of *H* between flying and mating males. *P* is one tailed, as discussed in the text.

expectation of evolutionary bioenergetics, that the scale of fitness consequences of genetic variation should covary positively with the magnitude of functional differences among genotypes (Watt 1985).

Because we have now found male mating success differences, roughly parallel in nature, among PGM genotypes in different *Colias* taxa, we must study functional differences among these genotypes. This will allow informed study of PGM allozymes' ecological interactions. PGM is a specific focus of organismal performance difference or natural selection in the wild in several other systems (e.g., Leigh Brown 1977; Keller and Ross 1993), and in one case a functional basis for this has been found (Pogson 1991). The mechanisms of these PGM male mating success effects must be quite different from those at PGI: PGM mediates carbon flow to and from middle-term glycogen storage, while PGI supports acute glycolytic response to flight energy demand from short-term trehalose/glucose reserves (cf. Watt and Boggs 1987; Carter and Watt 1988).

Systematics Implications of the Nonidentity of Allozymes Among Taxa

High-resolution electrophoresis shows that what appear, in ordinary electrophoresis, to be "the same" alleles of PGI in *C. meadii* as in lowland *Colias* are, in fact, different. Moreover, "similar" electromorphs have quite different values and rank orders of both thermal stability and kinetics among taxa. Coyne (1976) likewise found subtle electrophoretic differences among similar electromorphs of related *Drosophila,* though he did not compare them functionally.

For two decades, systematists of diverse groups have assessed phylogenetic relations of species within genera, or relations among genera, based on comparative electrophoretic analysis of allelic/genotypic identity and frequency among population samples from these taxa (e.g., summarized by Nei 1987, chapt. 9). Such work is usually done with ordinary starch-gel technique, and match of electromorph mobility in such conditions is taken as evidence of allelic identity, despite Coyne's (1976) implicit warning. The electrophoretic, functional, and fitness-related divergence of similar electromorphs in congeneric *Colias* reinforces the need for caution when concluding electrophoretic allele identity among *any* taxa. Systematic decisions made earlier on the basis of such conclusions may need re-evaluation with other character sources.

Adaptation and Constraint at the Molecular Level over Time and Among Taxa

To study the evolutionary history of *Colias*' PGI allozymes, we need a sequence-based allele phylogeny for them, within and among taxa. Such work has clarified the evolution of *Drosophila* alcohol dehydrogenase (e.g., Kreitman, Shorrocks, and Dytham 1992) and is now under way for *Colias* PGI (D. Pollock, unpublished). It will be informative to contrast such an allele phylogeny with well-understood taxon phylogeny for *Colias.* This is not yet available. *Colias* are morphologically diverse worldwide but with much parallel character variation, so that apart from subgeneric groupings, relations of clades above the level of species are obscure (e.g., Berger 1986). Molecular systematics, using genes independent of the present allozymes, may resolve these problems; this is now under way for *Colias* (D. Pollock, W. Watt et al., unpublished).

The PGI polymorphs of present *Colias* taxa may have diverged from an ancestral polymorphism while the organism–environment interactions of daughter taxa

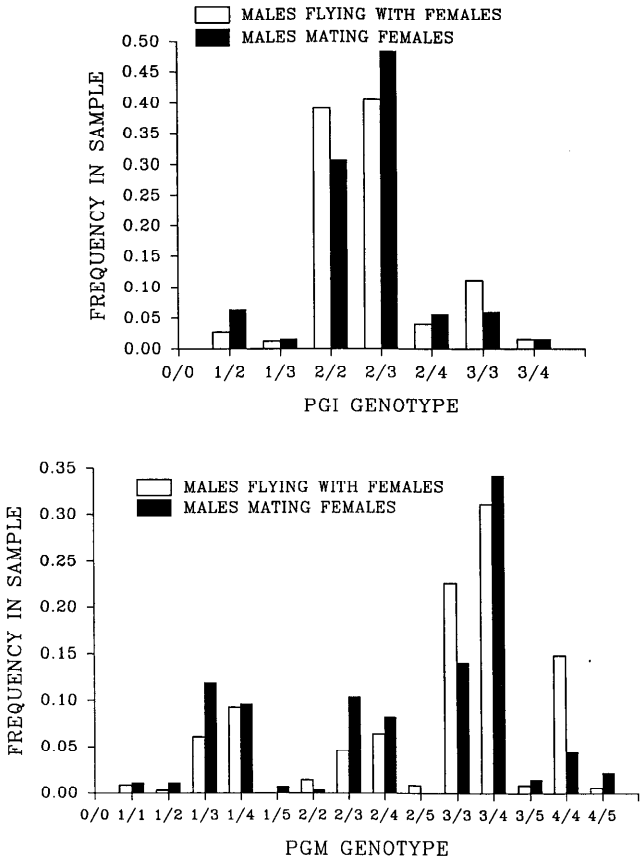

FIG. 4.—Genotype-by-genotype comparison, at PGI and PGM loci, of *C. meadii* males flying with females (open bars) vs. those successfully mating females (filled bars), in the pooled sample data of figure 3 and table 7. In each case, common heterozygotes are significantly advantaged and common homozygotes disadvantaged by a conservative G-test of frequency homogeneity: for PGI, $G = 16.045$, df $= 6$, $0.01 < P < 0.025$; for PGM, $G = 50.04$, df $= 13$, $P < 0.001$.

have themselves changed through evolution. Alternatively, the variants may be of independent, taxonomically parallel origin, which would imply an interesting degree of repeatability of evolutionary innovation. In either case, in perhaps a few million years of *Colias*' generic history in North America, in insects of large effective population size and wide geographic distribution (e.g., Hovanitz 1950; Watt et al. 1977), with the extended opportunity thus afforded for mutational "scanning" of their genomes, it appears that mutation has not found alleles which "optimize" functional properties when homozygous. This implies sharp constraints on the structural basis of PGI's function (cf. Gould 1980), such that maximal enzymatic performance can only be obtained by heterodimers, which arise in heterozygotes. Thus, by further study of this system, we can explore general evolutionary questions about the interactions of adaptation, constraint, and phyletic history in producing patterns of molecular evolution.

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