ADAPTATION AT SPECIFIC LOCI. II. DEMOGRAPHIC AND BIOCHEMICAL ELEMENTS IN THE MAINTENANCE OF THE COLIAS PGI POLYMORPHISM

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ABSTRACT

Demographically oriented sampling in the wild and biochemical study of allozymes in the laboratory have been used to probe maintenance of the phosphoglucose isomerase polymorphism of Colias butterflies.——The several alleles at this locus show negative or no covariation among their frequencies in the wild. This rules out Wahlund effects as a cause of observations of heterozygote excess at this locus in broods that fly as single cohorts. Unusually heavy mortality among adults, due to drought stress or other causes, can preclude manifestation of differential survivorship among phosphoglucose isomerase genotypes. In broods composed of overlapping cohorts, heterozygote deficiency, apparently due to Wahlund effects in time as cohorts of different survivorship experience mix, can be found. Heterozygotes at this locus fly under a broader range of weather conditions than other genotypes.——Previously detected kinetic differentiation among the genotypes extends in greater magnitude to the glycolytic reaction direction, as well as to a broader range of test conditions than examined before. The heterozygote 3/4 is strikingly heterotic for several measures of kinetic functional effectiveness. Other heterozygotes are sometimes heterotic, more often intermediate (but not exactly so, nor additive in any sense) in properties between homozygotes.——Predictions are made from the biochemical analysis and from the insects' thermal ecology concerning distributions of the genotypes in the wild. Some agree with facts already established. Others are tested and confirmed from data already on hand. Still others are to be tested as reported in an accompanying paper.——All available evidence points to a combination of heterozygote advantage and fluctuating-environment selection as responsible for maintaining this polymorphism. There is considerable evidence for the operation of protein-structural constraint on the range of adaptations possible at this locus.

AT the outset of a program to study the evolutionary meaning of natural variation in glycolytic enzymes, a polymorphism in phosphoglucose isomerase (PGI, EC 5.3.1.9) of Colias butterflies attached initial attention. This locus displays an association of allele frequency with habitat temperature. In populations that behave as single cohorts, it also shows the accumulation of striking

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heterozygote excess (up to 10–15% of the whole sample) with increasing age of the adults sampled (WATT 1977).

The latter finding at once suggested possible mechanisms for the maintenance of the polymorphism. It could, in principle, have been due to (1) some form(s) of natural selection acting on the PGI locus itself; (2) neutrality of PGI with selection on a closely linked, unknown locus (the "hitchhiking" effect); or (3) neutrality of PGI interacting with purely demographic effects.

Alternative 1 was supported by functional differences found among the genotypes at the biochemical level (WATT 1977). There are differences in thermal stability, up to four-fold, among genotypes of the four alleles so far studied (those frequent to common in lowland populations). Those alleles most frequent in the warmer habitats are most heat stable and vice versa. Further, major differences in maximum activity, in substrate affinity at low temperature, and in V_{max}/K_m ratio were seen when kinetics of enzymes produced by the different genotypes were studied in the gluconeogenic reaction direction (conversion of fructose-6-phosphate, F6P, to glucose-6-phosphate, G6P). These findings suggested possible selective differences among the genotypes: (1) kinetic superiority of certain heterozygotes might confer direct heterozygote advantage in fitness components related to glycolytic function; (2) genotypes of differing thermal stabilities might have very different fitnesses in populations exposed to different risks of environmental overheating stress; (3) the opposed directions of trends in stability and kinetic differences might confer net advantage on some heterozygotes in fluctuating environments, e.g., if greater thermal stability of a heterozygote's PGI resulted in persistence of greater functional capacity at the PGI step, under heat stress, as compared with a kinetically more effective, but less thermally stable, homozygote and a kinetically less effective, although somewhat more stable, homozygote.

Further study of this case seemed important for several reasons.

The initial purpose of the work was to probe the adaptive organization of glycolysis as a "model" metabolic system, with respect to effects of environmental temperature fluctuation, using just such polymorphisms as this one.

If previous indications of strong selection acting on this locus were supported by further work, this case would be firmly established among a few other developing case studies indicating strong selection on some allozymes (such as Drosophila alcohol dehydrogenase, e.g., VAN DELDEN, BOEREMA and KAMPING 1978; CAVENER and CLEGG 1978; coelenterate PGI, HOFFMANN 1981a,b; mouse hemoglobins, SNYDER 1981; fish LDH, PLACE and POWERS 1979; DIMICHELE and POWERS 1982a,b). This polymorphism, unlike the others, is multiallelic and, thus, might display a broader variety of selective differences.

If a selective explanation for this polymorphism were *not* sustained, the result would hardly be of less interest. LEWONTIN (1974) pointed out that sickle cell anemia was then our only well-documented case of heterozygote advantage. But, outside human populations (CAVALLI-SFORZA and BODMER 1971), *well-documented* cases of genetic drift effects are hardly more common. Perhaps the best is that of the *t* allele polymorphism of house mice (LEWONTIN and DUNN 1960; LEWONTIN 1962), which also involves a balance between gametic and zygotic selection.

Alternative 3 arose from the fact, pointed out by LI (1969) and MILKMAN (1975), that in a multiallele polymorphism positive correlation of allele frequencies is possible. If it does occur, and if subpopulations with different allele frequencies mix, a *positive* Wahlund effect results: apparent heterozygote excess is produced. Whether this effect was at work in the present case could be tested by examining patterns of population exchange of migrants, and by examining the sign and magnitude of allele frequency covariation. The basics of Colias demography are well understood (WATT *et al.* 1977; WATT, HAN and TABASHNIK 1979; TABASHNIK 1980). Demographically oriented genetic sampling to test alternative 3 would also allow examination of other demographic aspects of the polymorphism, e.g., interaction of the polymorphism with overlapping cohort population structure, and with extreme mortality unrelated to the PGI locus itself.

Alternatives 1 and 2 could be distinguished by two approaches. Exhaustive structural-genetic analysis of the chromosomal vicinity of the PGI locus could reveal whether any closely linked loci might be plausible "carriers" of hitchhiking PGI alleles. Even in so well characterized an organism as *Drosophila melanogaster,* this would be a formidable task. On the other hand, deeper biochemical study of Colias' PGI genotypes, especially of their kinetics in the glycolytic (G6P→F6P) reaction direction, could further test the match between these genotypes' functional properties and their distributions in wild populations. Predictions about unexplored aspects of the genotypes' field distributions could then be made and tested. Since this latter route would explore the adaptive organization of glycolysis at the same time, as originally planned, it was adopted.

A comprehensive analysis of enzyme function in evolutionary terms is beyond the scope of the present paper. But, some discussion of the choice of biochemical criteria for adaptiveness (or its absence) of PGI genotypic differences, and the range of pH, temperature, etc. conditions appropriate for study, is needed. It follows this paper as APPENDIX I. The following are its conclusions and are the fitness indices of biochemical function used hereafter.

1. Values of realized glycolytic flux, in steady state or in transient state, can limit Colias' capacity for flight activity and, hence, affect the components of fitness discussed later.

2. The PGI step's flux capacity in Colias is selected to display a *low* sensitivity coefficient (KACSER and BURNS 1973, 1979) toward glycolytic system flux so as to *minimize* the PGI step's influence on system transient response and steady state flux capacity, thus maximizing the control of system transients or steady state flux by allosteric enzymes (such as phosphofructokinase) in response to energy demands signaled by energy charge fluctuations (ATKINSON 1977).

The genotype-specific K_ms, V_{max}/K_m ratio and thermal stabilities measurable on Colias PGI allozymes *in vitro* directly reflect differences in kinetic performance of this step and the enzyme's stability *in vivo.*

4. Small K_m, large V_{max}/K_m and high thermal stability values are different phenotypes positively selected to minimize the PGI step's sensitivity coefficient toward glycolytic flux and, thus, to maximize glycolytic flux response and capacity, especially under suboptimal or stressful thermal conditions.

MATERIALS AND TECHNIQUES

C. philodice eriphyle Edwards was sampled from 1700 m (5600 ft) elevation outside Olathe, Montrose County, Colorado, up to 2710 m (8900 ft) elevation near Crested Butte Mountain, Gunnison County, Colorado (a site of previous Colias PGI sampling). *C. meadii* Edwards was sampled at 3350, 3540 and 3720 m (11,000, 11,500 and 12,000 ft) on the Mesa Seco, Hinsdale County, Colorado, and at 3690 m (12,100 ft) on Cumberland Pass, Gunnison County, CO.

Except in a few cases, population samples to be compared were taken at the same time of day. This was done at first as a precaution, but it proved important, as will be seen. Wing wear ratings for indexing the age of sampled animals were assigned in the manner of WATT, HAN and TABASHNIK (1979), as explained here in the caption to Table 1.

Discontinuous polyacrylamide gel electrophoresis for genotyping sampled animals was done as before (WATT 1977), save that BioRad slab gel apparatus was used.

The nomenclature of the PGI alleles has been changed; they are now numbered in order of increasing electrophoretic mobility, rather than lettered. Old and new designations are: $T' = 1$, $T = 2$, $S = 3$, $M = 4$, $F = 5$, $F' = 6$. An even faster allele, 7, having a mobility of 1.57 relative to horse ferritin in the Tris-glycine buffer system of DAVIS (1964) and gels of 8% T, 0.4% C (RODBARD and CHRAMBACH 1971) has been seen once, in a heterozygote of *C. p. eriphyle*.

PGI of the different genotypes was prepared from our laboratory colony of *C. eurytheme* Boisduval exactly as before, except that purification is now routinely carried out through the DEAE-Sephadex A25 ion exchange step (WATT 1977) for purposes of standardization, even though no detectable difference in kinetics can be found between enzyme preparations before and after the step. (Other proteins potentially competing for substrate or product were removed earlier.) The final purification resulting is 250–300×, in 65% yield, as compared with crude homogenates.

DYSON and NOLTMANN'S (1965) assay for PGI in the glycolytic direction uses phosphofructokinase (PFK) as a coupling enzyme and titrates, in a pH stat, the protons released by the splitting of adenosine triphosphate (ATP) as PFK phosphorylates F6P, the product of PGI in the glycolytic direction, in order to track the PGI reaction itself. A Radiometer TTT2 autotitrator, ABU12 autoburette, SBR3 recorder, TTA31 semimicroreaction vessel with thermostat jacket, HETO thermocirculator and ice bath exchange coil and a GK3230C miniature combination electrode were used for the pH stat assays. NaOH, 2.5×10^{-4} M was used as titrant, and all reactions were run under a nitrogen flush (500 ml/min) to exclude the atmospheric CO_2 blank. Results were corrected for minor variation in titrant strength, as monitored with standardized potassium hydrogen phthalate solutions.

All pH measurements were done with ceramic junction electrodes, to avoid the errors resulting from reaction of Tris buffers with fiber junctions. The temperature coefficient of Tris' ionization was explicitly accounted for in all cases.

The basis of pH-stat reaction solutions is a mixture of 0.05 M KCl, 0.002 M K_2SO_4 (sulfate acts as an analog of inorganic phosphate for activation of PFK, while avoiding mixed salt precipitations characteristic of phosphate; LOWRY and PASSONNEAU 1966), 0.002 M $MgCl_2$ (magnesium is required in excess of ATP for formation of the actual PFK cosubstrate MgATP, and is also a PFK activator; LOWRY and PASSONNEAU 1966) 0.02% NaN_3 (antibacterial) and 0.001 M dithiothreitol (DTT) (antioxidant). Buffering ions are excluded. Great care must be taken to exclude calcium, as this cation is a strong negative effector of PFK; this runs to the extent of storing reagents in a silica gel-charged desiccator, rather than one charged with the more usual $CaCl_2$.

The following are cosubstrates and activators of the coupling enzyme: 2×10^{-4} M ATP, 5×10^{-4} M 3′,5′-cyclic AMP (cAMP), 2×10^{-5} M fructose-1,6-diphosphate (FDP).

Rabbit muscle phosphofructokinase (Sigma Chemical Company) was rehydrated from crystalline suspension in ammonium sulfate, passed over a column of Sephadex G25 to remove ammonium ions and buffering ions in the presence of 1×10^{-4} M ATP, 2×10^{-4} M AMP and 0.001 M DTT as stabilizers, then stored under nitrogen until it was used as coupling enzyme at 3 EC units/1.5 ml assay reaction mixture.

Assays for kinetic parameter determinations on PGI were run in duplicate at 75, 150, 300, 600 and 1200 μM concentrations of G6P substrate at pH 8.0 and 8.75; 37.5, 75, 150, 300, and 600 μM were used at pH 7.25. These values were chosen to ensure adequate bracketing of the actual K_m values by the experimental concentrations. Although inhibition of PGI by high concentrations of ATP and related compounds has been reported for vertebrate PGI (KAHANA *et al.* 1960), no such effect can be

found in Colias PGI with respect to ATP concentrations used here. This may be related to the somewhat tighter substrate binding characteristic of mammalian PGI (KAHANA *et al.* 1960) or to the presence of magnesium as suggested by several authors. As before, K_m values were found to be the same among different enzyme "batches" of the same genotype, often purified a year or more apart.

V_{max} ratio determinations were done in triplicate for each assay direction on the same dilutions of the same enzyme preparations; all assays were completed in both directions within 10 min for each determination. Here again, these values were quite independent, within genotypes, of the specific enzyme preparations used.

Data reduction and statistical testing were done in standard fashion (GOLDSTEIN 1964; SOKAL and ROHLF 1969; ROHLF and SOKAL 1969), using CCS, Compupro and Data General computers.

RESULTS

Demographic aspects

Allelic covariation and a test of alternative 3: Mixing of populations with distinct allele frequencies can result in heterozygote excess due to a Wahlund effect, as noted, only if multiallelism allows positive covariation of allelele frequencies (LI 1969; MILKMAN 1975). In Colias, the two most common alleles are of particular interest, as the most common heterozygote contributes at least 70–80%, often more, of heterozygote excess when this is observed (WATT 1977).

Table 1 presents allele frequency data for Gunnison Basin populations of *C. p. eriphyle* and *C. meadii*. These data do not, and should not, show an overall trend of heterozygote excess; many of the samples are not from single cohorts, many are composed of young animals, etc. Examination of the variation and covariation of allele frequencies was the purpose of this sampling; further documentation of the already demonstrated (WATT 1977) accumulation of heterozygote excess with age in single-cohort broods would have dictated other sampling patterns.

Fractions of population members dispersing, and their dispersal radii, are known for these species (WATT *et al.* 1977; WATT, HAN and TABASHNIK 1979). These data and the observed range of allele frequencies do not fit with a Wahlund effect as a source of heterozygote excess. For example, assuming a mixture wherein 50% of the individuals are drawn from each of the two most different *C. p. eriphyle* samples (12 and 15, Table 1), a total heterozygote *deficiency* of −2.4% results. But the samples in question are from habitats many kilometers distant, and the average dispersal radius of this species is on the order of 0.3 km, so such extended mixing of populations simply does not occur.

An even more definitive test of alternative 3 is possible, however. We can treat samples *as if they were* from subpopulations that routinely exchange large numbers of migrants and evaluate allelic covariation among them. Alternative 3 would be tenable under *any* migration exchange conditions if and only if positive covariation occurs among those alleles whose genotypes develop heterozygote excess with age in single cohorts.

Table 2 presents allelic variances and covariances calculated after LI (1969) for all samples of both species. For both, allelic covariances between the two most common alleles (*2* and *3* in *C. meadii*) are strongly *negative*. No other allelic combinations show major covariation, and most of the coefficients are negative in any case. These data cannot be evaluated using a standard correla-

TABLE 1

Primary wild-sample data on Colias PGI for evaluation of allele covariation and other purposes

Sample	n	$\bar{R}$	H_{exc}	M_{exc}	p_1	p_2	p_3	p_4	p_5	p_6	Comments if any	Brood
Colias p. eriphyle												
1. 19 Aug 76 10:30–11:15 C.B. Mtn, CO (05.06)	53	2.18	+0.003	+0.015	0	0.047	0.623	0.245	0.076	0.009		1976II
2. 28 Aug 76 10:40–11:15 C.B. Mtn, CO (04,10)	60	2.35	+0.026	+0.026	0.008	0.050	0.658	0.233	0.050	0		1976II
3. 4 Sept 76 10:30–11:00 C.B. Mtn, CO (02,03)	47	3.01	+0.048	+0.041	0	0.021	0.596	0.340	0.043	0		1976II
4. 2 July 77 10:30–12:00 C.B. Mtn, CO (02,04,06)	57	3.94	+0.089	+0.081	0.009	0.061	0.658	0.272	0	0		1977I
5. 19 Aug 77 11:30–12:00 C.B. Mtn, CO (02,08)	45	2.39	−0.018	−0.035	0	0.078	0.678	0.222	0.022	0		1977II
6. 30 Aug 77 10:50–11:40 C.B. Mtn, CO (05)	53	2.44	−0.081	−0.011	0.009	0.142	0.519	0.283	0.047	0	Clear sunny weather (*cf.* #14)	1977II
7. 5 Sept 77 1300–1400 C.B. Mtn, CO (04,05)	80	2.75	+0.001	+0.003	0.006	0.038	0.669	0.250	0.031	0.006		1977II
8. 7 July 78 10:30–12:05 C.B. Mtn, CO (01–03)	53	3.55	+0.025	+0.032	0	0.076	0.604	0.255	0.066	0	Not end of brood sample; extremely low density for brood I	1978I
9. 23 Aug 1978 10:00–12:05 C.B. Mtn, CO (05–06)	40	2.29	+0.063	+0.035	0.013	0.088	0.600	0.263	0.038	0	Extremely low density for brood II	1978II
10. 2 Aug 77 10:30–12:00 Cimarron, CO	53	2.76	+0.007	+0.034	0	0.038	0.594	0.321	0.047	0	71 km from C.B. Mtn	1977II
11. 10 Aug 77 10:30–12:00 Olathe, CO	40	3.30	+0.001	+0.035	0	0.063	0.663	0.238	0.038	0	94 km from C.B. Mtn	1977II
12. 24 Aug 77 12:00–12:40 Gunnison, CO	47	2.59	−0.006	−0.003	0	0.075	0.692	0.202	0.032	0	36 km from C.B. Mtn	1977II
13. 29 Aug 77 13:10–14:00 Jack's Cabin, CO	45	2.50	+0.039	+0.014	0.011	0.111	0.589	0.233	0.056	0	18 km from C.B. Mtn	1977II
14. 30 Aug 77 12:05–12:40 Slate Corner, CO	54	2.48	+0.023	−0.002	0	0.083	0.546	0.324	0.037	0	Partly cloudy; deteriorating to rain; *cf.* #6; 3/7 heterozygote in this sample; 2.5 km from C.B. Mtn	1977II

15. 27 July 80 10:30–12:00 Olathe, CO	40	2.48	+0.014	−0.002	0	0.075	0.488	0.413	0.025	0	Midst of unusual hot spell; 94 km from C.B. Mtn.	1980II
Colias meadii												
16. 9 July 77 10:15–11:00 Mesa Seco, CO (02)	40	2.60	+0.018	+0.013	0.038	0.500	0.438	0.025	0	0	Clear sunny weather (*cf.* #17)	N/A
17. 9 July 77 11:00–11:45 Mesa Seco, CO (05)	40	2.65	+0.042	+0.036	0.025	0.488	0.450	0.038	0	0	Partly cloudy, deteriorating to rain (*cf.* #16)	N/A
18. 25 July 77 10:30–11:30 Mesa Seco, CO (10)	40	2.51	+0.022	+0.019	0.025	0.625	0.325	0.025	0	0	Clear	
19. 19 July 78 9:55–10:50 Mesa Seco, CO (02)	40	2.26	+0.032	+0.013	0.013	0.575	0.350	0.063	0	0	Clear, sunny (*cf.* #20)	N/A
20. 19 July 78 11:10–12:20 Mesa Seco, CO (05)	45	2.27	+0.082	+0.079	0.022	0.511	0.444	0.022	0	0	Partly cloudy, interspersed w/ showers (*cf.* #19)	N/A
21. 24 July 78 9:45–10:30 Mesa Seco, CO (10)	44	2.50	+0.023	+0.020	0.011	0.625	0.330	0.034	0	0	Clear	N/A
22. 7 Aug 78 10:15–11:30 Cumberland Pass, CO (01)	46	3.39	+0.070	+0.067	0.033	0.663	0.294	0.011	0	0	Clear, deteriorating to partly cloudy	N/A

C.B. Mtn = Crested Butte Mountain; n = number of animals per sample; $\bar{R}$ = average wing wear rating as index of age, overall scale from 1 (wings not yet dry) to 5 (scales extremely eroded and wing cuticle extremely damaged), following WATT, HAN and TABASHNIK (1979); H_{exc} = excess of total heterozygote frequency over that expected from a multiallelic Hardy-Weinberg distribution of the allele frequencies (as fraction of whole sample); M_{exc} = excess of most common heterozygote (*3/4* for *C. p. eriphyle*; *2/3* for *C. meadii*) over Hardy-Weinberg expectation (as fraction of whole sample); p_i = frequency of the ith allele. Parenthetic numbers following localities are subsites within the overall study areas.

TABLE 2

Allelic covariation in samples of Table 1

Comparisons	n	i	$\bar{p}_i$	j	$\sigma^2 p_i$ or $\sigma p_i p_i$	τ	P (two-tailed)
C. p. eriphyle, all samples	15	2	0.070		+0.00065		
C. p. eriphyle, all samples	15	2		3	−0.00169	−0.174	$0.3 < P < 0.4$
C. p. eriphyle, all samples	15	2		4	−0.00075	−0.116	$0.5 < P < 0.6$
C. p. eriphyle, all samples	15	2		5	−0.05005	+0.049	$0.8 < P < 0.9$
C. p. eriphyle, all samples	15	3	0.612		+0.00699		
C. p. eriphyle, all samples	15	3		4	−0.00244	−0.596	$P = 0.002$
C. p. eriphyle, all samples	15	3		5	−0.00012	−0.183	$0.3 < P < 0.4$
C. p. eriphyle, all samples	15	4	0.273		+0.00157		
C. p. eriphyle, all samples	15	4		5	−0.00021	−0.087	$0.6 < P < 0.7$
C. p. eriphyle, all samples	15	5	0.041		+0.00033		
C. p. eriphyle, CB Mtn samples	9	3	0.623		+0.00646		
C. p. eriphyle, CB Mtn samples	9	3		4	−0.00075	−0.620	$0.01 < P < 0.05$
C. p. eriphyle, CB Mtn samples	9	4	0.262		+0.00025		
All other covariation τs insignificant							
C. p. eriphyle, all except CB Mtn	6	3	0.595		+0.00409		
C. p. eriphyle, all except CB Mtn	6	3		4	−0.00409	−0.733	$0.05 < P < 0.10$
C. p. eriphyle, all except CB Mtn	6	4	0.289		+0.00487		
All other covariation τs insignificant							
C. meadii, all samples	7	1	0.024		+0.00008		
C. meadii, all samples	7	1		2	−0.00007	−0.10	$0.05 < P$
C. meadii, all samples	7	1		3	+0.00008	+0.048	$0.05 < P$
C. meadii, all samples	7	1		4	−0.00010	−0.40	$0.05 < P$
C. meadii, all samples	7	2	0.570		+0.00621		
C. meadii, all samples	7	2		3	−0.00401	−0.927	$P = 0.01$
C. meadii, all samples	7	2		4	−0.00048	−0.40	$0.05 < P$
C. meadii, all samples	7	3	0.376		+0.00293		
C. meadii, all samples	7	3		4	−0.00014	+0.293	$0.05 < P$
C. meadii, all samples	7	4	0.031		+0.0002		

n = number of samples in comparison; i,j = allele designators; $\bar{p}_i$ = mean frequency of allele i in samples; $\sigma^2 p_i$ = variance of p_i frequency; $\sigma p_i p_j$ = covariance of p_i and p_j frequencies; τ = Kendall's coefficient of rank correlation. Rare alleles, $p_i < 0.01$, have not been included in this analysis.

tion coefficient since, as LI (1969) points out, this is not well defined for multiallele polymorphisms. But Kendall's rank correlation coefficient does not suffer in this way. The only significant correlations are highly negative ones between alleles 3 and 4 in *eriphyle,* alleles 2 and 3 in *meadii.*

The data base for *eriphyle* allows examination of some meaningful parts of it. For example, all samples taken at Crested Butte Mountain through time show negative covariance and significant negative correlation of allelic ranks for alleles 3 and 4 (Table 2). Samples from *all other* Gunnison Basin sites do likewise except that the negative rank correlation is not quite significant. Other, even smaller subsets of the samples, such as those taken at different points in the East River drainage within 6 days (samples 6 and 12–14), etc., uniformly show negative covariation among these allele frequencies, although significance evaluation is prevented by the small number of samples.

From these results, there remains no possibility that a Wahlund effect can underlie the accumulation of heterozygote excess with age in single cohorts previously observed. Population mixing on any scale can only produce heterozygote deficiency, or else no effect at all, and alternative 3 is rejected decisively.

Extreme mortality and the PGI polymorphism: Above 2100 m elevation in Gunnison County, Colorado, *C. p. eriphyle* has two broods. The first flies as a single cohort and is usually well supplied with flower nectar as food. However, the second brood not only shows extensive overlap of cohort structure, flying for as many as 7 weeks, but at the upper limits of its range at Crested Butte Mountain is frequently nectar limited, due to late summer dryness and/or early flower senescence. First broods, if numerous enough to sample at their close, always show heterozygote excess, agreeing with the earlier demonstration that such excess accumulates with age in such broods (e.g., sample 4 of Table 1; this sample by itself is too small for statistical significance, but the value itself is just as expected). Second broods, at Crested Butte Mountain, however, frequently show little or no accumulation of heterozygote excess (Table 1: brood 1976II, samples 1–3; brood 1977II, samples 5 and 6).

One major contributor to this effect, or lack of one, seems to be unusually high mortality for Colias populations. In both these broods, unusually low nectar availability led to very high measured mortality (WATT, HAN and TABASHNIK 1979; similar drought effects on mortality through nectar supply have been seen in other Colias populations by WATT *et al.* 1977). Estimates of *daily* loss rates in this population were: brood 1976II, males 0.285, females 0.582, and brood 1977II, males 0.358, females 0.311. Under such conditions, more than half the adult population does not live to reach sexual maturity. This high mortality was also evidenced by failure of the flying populations to accumulate the wing wear characteristic of normally aging adults. For example, on our wing wear aging scale of 1 to 5 (caption to Table 1; WATT, HAN and Tabashnik 1979), normal end-of-brood average values are displayed by brood 1977I—males 4.52, females 4.20—but our last sample for brood 1977II, after many weeks of flight, showed average values for males of 2.36 and for females of 2.57. The animals were simply dying before they could age appreciably. Such effects would certainly operate to forestall the appearance of differential survivorship on the

part of the PGI genotypes, at least on the scale routinely evidenced in first broods of *C. p. eriphyle*.

Apparent Wahlund effect in time: Samples 5 and 6 of Table 1 hint at the development of a slight heterozygote *deficiency* with time in brood 1977II, although this is not significant by a test for difference of percentages (GOLDSTEIN 1964). There is a potential mechanism for such an effect in second (or later, when these occur) broods. Since Colias PGI allele frequencies covary negatively and these later broods have overlapping cohort structure, there might develop a Wahlund effect over time in a population at one place, if the overlapping cohorts experienced different selection regimes due to change in weather or other effects, or even emerged with different starting allele frequencies due to differential oviposition of genotypes through the previous generation.

Brood 1980II was sampled at Gunnison, as reported following WATT, CASSIN and SWAN (1983), for the testing of predictions made later in the present paper. This sampling of an overlapping cohort brood also allowed search for temporal Wahlund effects, not masked by the extreme mortality common in brood II of the range-edge Crested Butte Mountain population. The sampling site is at the center of a very large population occupying several square miles of relatively uniform moist grassland. Densities of this brood were so high as to preclude *any* effect of initial sampling on results of later sampling. The population was sampled twice, on August 11 during prolonged, unusually hot weather, and again on August 26, just after the end of this "hot spell."

The overall sampling design is elaborate and will be fully discussed later (WATT, CASSIN and SWAN 1983). We can either compare the aggregate samples taken on each day or the "subsamples," C and G, respectively, taken at peak flight density on the 2 days. In each case, we can examine overall heterozygosity, and more specifically the two most common alleles, *3* and *4*, and their heterozygote *3/4*.

Usual mortality levels were clearly operating in the population, from the changes in average wing wear between the first and second sampling days: August 11, whole sample ($N = 229$) 2.82, peak subsample (C; $n = 62$) 2.90; August 26, whole sample ($N = 237$) 3.77, peak subsample (G; $n = 58$) 3.75. On the expectation of a temporal Wahlund effect, we predict a heterozygote deficiency in the late sample(s) as compared with the early; no effect or a "significant" heterozygote excess are equally violations of the expectation, so one-tailed tests are appropriate and necessary. Table 3 presents the summarized data and their evaluation by GOLDSTEIN's (1964) binomial tests for difference of two percentages or of one percentage from an expected value. For August 11, both composite sample and peak density subsample fail to deviate significantly from Hardy-Weinberg expectations, either in overall heterozygosity or in heterozygosity for the *3/4* genotype, whereas on August 26 both *do* deviate significantly and negatively at both levels of genotype resolution. Moreover, the early and late composite samples, and early and late peak subsamples, both differ very significantly from one another in percent deviation of overall and of *3/4* heterozygosity, away from Hardy-Weinberg expectations—again, with deficiency later in the season.

TABLE 3

Significance testing of early and late samples in brood 1980II of C. p. eriphyle *for departure of heterozygote frequency from Hardy-Weinberg expectations and from one another*

Comparison	diff (%)	n	x*	P (one-tailed)
Composite samples				
Overall heterozygosity				
A-D *vs.* H-W	+0.35	229	0.11	>0.9
E-H *vs.* H-W	−5.47	237	1.81	0.036
A-D *vs.* E-H	−5.82	229, 237	3.95	<0.0001
3/4 heterozygosity				
A-D *vs.* H-W	+1.33	229	0.42	0.33
E-H *vs.* H-W	−5.48	237	1.74	0.041
A-D *vs.* E-H	−6.81	229, 237	5.16	<0.0001
Peak subsamples				
Overall heterozygosity				
C *vs.* H-W	−1.29	62	0.21	0.41
G *vs.* H-W	−13.8	58	2.11	0.017
C *vs.* G	−12.5	62, 58	2.59	0.0048
3/4 heterozygosity				
C *vs.* H-W	−2.42	62	0.39	0.34
G *vs.* H-W	−11.3	58	1.75	0.040
C *vs.* G	−8.88	62, 58	1.93	0.027

Consult text for discussion of sampling protocol. A-D = composite sample of August 11, 1980; E-H = composite sample of August 26, 1980; C = peak density subsample of August 11, 1980; G = peak density subsample of August 26, 1980. H-W = Hardy-Weinberg expectation. Tests were GOLDSTEIN's (1964) binomial test for difference (dif) of a percentage from expected value (tests "vs. H-W"), or for difference (dif) of two observed percentages. One-tailed significance criterion justified in text.

A Wahlund effect, of the kind predicted, developed in brood 1980II. It cannot have been due to mixing by spatial dispersal. Given its geography, and the relatively short dispersal radius of *C. p. eriphyle* (WATT, HAN and TABASHNIK 1979; TABASHNIK 1980), the Gunnison sampling site is one neighborhood within a large surrounding population of extremely similar habitat, so that much allele frequency divergence would not be expected. Even the most divergent allele frequencies seen between separate localities in this species (see previously) would not produce such a large heterozygote deficiency. A *temporal* Wahlund effect, in place, must have occurred. The causes will be analyzed further later (WATT, CASSIN and SWAN 1983).

Apparent diurnal variation in genotype frequency: Once in *C. p. eriphyle*—sample 6 *vs.* sample 14, Table 1—and twice in *C. meadii—sample 16 vs.* sample 17, sample 19 *vs.* sample 20—samples of Colias PGI genotypes were taken at adjacent sample sites, in extremely similar habitats, with similar population densities and ages, but at different times on the same day. In all cases, the first sample was taken in clear sunny weather, the second in colder partial cloudiness, and the second sample showed greater PGI heterozygote excess over Hardy-Weinberg expectations than did the first sample.

These data can be tested as a 3 × 2 contingency table using the G test. The null hypothesis is that the data are random variations around their own Hardy-Weinberg expectations; the alternative is that the partly cloudy, colder weather samples have disproportionately greater heterozygosity in relation to their Hardy-Weinberg expectations than do the clear, sunny weather samples. Expected values for the table are given by the Hardy-Weinberg expectations themselves. The alternative is significant: $G = 9.87$, $d.f. = 2$, $0.005 < P < 0.01$. Colias PGI heterozygotes are active over a broader span of environmental conditions, notably thermal fluctuation, than other genotypes at this locus.

Biochemical aspects

Substrate binding properties of PGI genotypes: Table 4 presents K_m data for all ten genotypes, for both substrates, at pH 8.75 and temperatures of 10°, 30° and 40°. Table 5 presents analogous data for genotypes *3/3, 3/4 and 4/4* at all three temperatures at pH 8.0, and at 30° and pH 7.25. Figure 1 illustrates the values of K_m G6P for *3/3, 3/4* and *4/4* under all test conditions.

Genotypic differences in substrate binding are similar for both reaction directions—a genotype with relatively strong affinity, *i.e.*, low K_m, for F6P has strong affinity for G6P as well, and conversely for weak binding. These differences are *far more pronounced* in the glycolytic direction, *i.e.*, in K_m for G6P, than those previously reported for F6P. Analyses of variance for G6P K_ms show highly significant differences, without exception. For the most common heterozygote, *3/4*, heterotic advantage in K_m for G6P is two- to three-fold.

Temperature variation interacts sharply with the genotypic differences. All ten genotypes show a minimum in K_m for F6P at intermediate temperature. Some, *e.g., 2/2, 3/4, 4/4*, also show this in K_m for G6P, but others do not. Most heterozygotes are heterotic for K_m at low temperature—*4/5* is the exception—and again this is more dramatic in the glycolytic direction. Only *3/4* remains heterotic for K_m under all conditions studied. Other heterozygotes for the most part show intermediacy between their homozygotes, with some regaining moderate heterosis (*2/4, 2/5*) or else deteriorating altogether (*3/5*) at high temperature. The *3/3-3/4-4/4* subset tends to show asymmetry of heterosis, with *3/3* being closer to *3/4* in substrate affinity than is *4/4*.

As pH decreases, so do values of K_m, indicating tighter substrate binding and greater ease of saturation. Genotypic differences persist in kind and may become even more pronounced, as preliminary data had suggested. There is an overall gain in relative saturability for G6P *vs.* F6P as pH decreases, so that at pH 7.25 the *3/4* genotype is equally saturable by either substrate at 30°.

Equilibrium relations and V_{max} ratios of the PGI step: The values of V_{max} and K_m for this reversible reaction are mutually constrained by the equilibrium constant through the Haldane equation (APPENDIX I, equation 2). Values of K_{eq} at relevant temperatures and pHs are of interest in their own right. They can also be used as a check on the internal consistency of the data base. K_ms and V_{max} ratios for certain genotypes were taken a year or more apart and could have been subject to error by unwitting variation of technique over that time. If so, divergent values of K_{eq} should be produced by Haldane equation calculations for the several genotypes. Agreement of K_{eq} values, calculated by the

TABLE 4

Colias *PGI* K_ms *for F6P and G6P at pH 8.75*

		Genotypes									
		2/2	*2/3*	*3/3*	*3/4*	*4/4*	*4/5*	*5/5*	*2/4*	*2/5*	*3/5*
10°	F6P	124 ± 4	116 ± 4	112 ± 6	110 ± 5	123 ± 4	137 ± 4	130 ± 2	91 ± 5	105 ± 6	105 ± 6
	n	3	3	3	4	4	3	5	4	4	4
	G6P	166 ± 17	130 ± 6	171 ± 10	137 ± 10	282 ± 10	221 ± 24	189 ± 8	73 ± 10	138 ± 17	86 ± 10
	n	4	4	4	5	4	4	3	4	5	3
	Ratio	0.747	0.892	0.655	0.803	0.436	0.620	0.688	1.247	0.761	1.221
30°	F6P	66 ± 4	72 ± 3	76 ± 4	68 ± 1	76 ± 7	74 ± 1	65 ± 2	65 ± 5	70 ± 6	76 ± 5
	n	4	3	3	3	4	3	4	3	3	3
	G6P	142 ± 7	215 ± 13	185 ± 7	125 ± 9	211 ± 16	208 ± 11	163 ± 12	154 ± 17	170 ± 12	126 ± 9
	n	5	6	9	6	8	4	5	5	5	4
	Ratio	0.465	0.335	0.411	0.544	0.360	0.356	0.399	0.422	0.411	0.603
40°	F6P	114 ± 6	125 ± 3	135 ± 14	114 ± 7	118 ± 7	125 ± 5	118 ± 9	109 ± 3	131 ± 13	118 ± 4
	n	4	4	4	4	4	4	4	4	4	4
	G6P	337 ± 15	305 ± 21	294 ± 17	174 ± 13	353 ± 23	325 ± 35	332 ± 9	293 ± 18	243 ± 26	427 ± 18
	n	9	5	7	6	7	5	7	5	5	5
	Ratio	0.338	0.410	0.459	0.655	0.334	0.385	0.355	0.372	0.539	0.276

Data tabled are means ± standard errors of means. All K_m values are in units of micromolar substrate concentration. Analysis of variance significance testing of K_m differences: F6P 10°, $F_{9,27} = 9.39$, $P < 0.001$; G6P 10°, $F_{9,30} = 19.1$, $P < 0.001$; F6P 30°, $F_{9,23} = 1.12$, $0.25 < P < 0.50$; G6P 30°, $F_{9,47} = 7.51$, $P < 0.001$; F6P 40°, $F_{9,30} = 1.0$, $0.25 < P < 0.50$; G6P 40°, $F_{9,51} = 11.07$, $P < 0.001$.

TABLE 5

Colias *PGI* K_ms *for genotypes* 3/3, 3/4, 4/4 *at low pH*

		Genotypes		
		3/3	3/4	4/4
pH 8.00				
10°	F6P	151 ± 10	125 ± 4	156 ± 4
	n	5	4	5
	G6P	178 ± 14	100 ± 6	307 ± 35
	n	5	5	5
	Ratio	0.848	1.250	0.508
30°	F6P	102 ± 6	76 ± 6	97 ± 4
	n	5	5	5
	G6P	192 ± 18	114 ± 9	190 ± 27
	n	5	5	5
	Ratio	0.531	0.667	0.511
40°	F6P	93 ± 7	79 ± 12	104 ± 10
	n	5	4	4
	G6P	272 ± 47	147 ± 20	296 ± 46
	n	5	5	5
	Ratio	0.342	0.537	0.351
pH 7.25				
30°	F6P	114 ± 7	108 ± 7	129 ± 11
	n	5	5	5
	G6P	181 ± 19	105 ± 13	192 ± 19
	n	7	5	5
	Ratio	0.630	1.03	0.672

Data tabled are means ± standard errors of means. Units of K_m are micromolar substrate concentration. *n* = number of determinations averaged to give each K_m estimate. Analysis of variance significance testing of K_m differences: pH 8.00, 10°, F6P $F_{2,11} = 5.11$, $0.025 < P < 0.05$, G6P $F_{2,12} = 22.57$, $P < 0.001$; 30°, F6P $F_{2,12} = 32.47$, $P < 0.001$, G6P $F_{2,12} = 25.96$, $P < 0.001$; 40°, F6P $F_{2,10} = 1.61$, $0.10 < P < 0.25$, G6P $F_{2,12} = 20.48$, $P < 0.001$; pH 7.25, F6P $F_{2,12} = 1.59$, $0.10 < P < 0.25$, G6P $F_{2,14} = 6.49$, $P = 0.01$.

Haldane method, among genotypes would show consistency of these data, since K_{eq} is a thermodynamic property of F6P and G6P themselves, and should not be affected by genotypic changes.

K_{eq} estimates were made, given the K_m data, by careful determination of V_{max} ratios (see later) and substitution into (2). Results are shown in Table 6; K_{eq} is closer to 1, favoring flux in the glycolytic direction, at lower temperatures and pHs.

KAHANA *et al.* (1960) and DYSON and NOLTMANN (1968) asserted, without furnishing data, that K_{eq} for the PGI reaction does not vary "significantly" with pH. However, DYSON and NOLTMANN proposed a chemical mechanism for the reaction involving addition of a proton from surrounding solution as the last step in F6P formation— therefore, the loss of this proton as the first step in F6P conversion to G6P. This should render the balance of the reaction at least somewhat pH sensitive, with F6P being less stable at higher pH. The decrease in K_{eq} at higher pH found here is not massive but repeatable and consistent with this proposed mechanism.

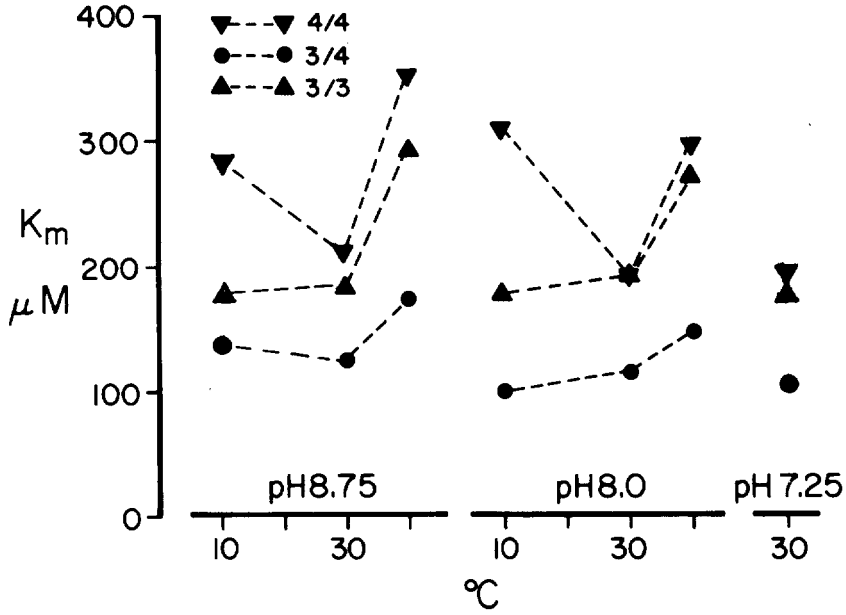

FIGURE 1.—Substrate binding affinity for G6P, as indexed by K_m, of the three most common Colias PGI genotypes. Means are given; for error measures and statistical testing (genotypic differences are highly significant), see Tables 1 and 2. Recall that *low* K_m means *strong* substrate affinity and, conversely, *high* K_m means *weak* substrate affinity.

TABLE 6

Values for equilibrium constant of the PGI reaction

	Genotypes				
	2/2	2/3	3/3	3/4	4/4
pH 8.75					
10°	0.68	0.69 ± 0.01	0.69 ± 0.07	0.71 ± 0.06	
n	1	2	2	1	
30°	0.215 ± 0.002	0.217 ± 0.001	0.216 ± 0.006	0.218 ± 0.004	0.212 ± 0.002
n	2	3	5	2	4
40°	0.186	0.195	0.202 ± 0.007	0.194 ± 0.002	0.193 ± 0.005
n	1	1	4	2	4
pH 8.00					
10°			0.85 ± 0.020	0.93 ± 0.02	0.83 ± 0.06
n			2	2	2
30°			0.33 ± 0.03	0.37 ± 0.02	0.34 ± 0.04
n			3	6	5
40°			0.30 ± 0.001	0.33 ± 0.01	0.34 ± 0.03
n			2	2	2
pH 7.25					
30°			0.33	0.35 ± 0.03	0.34
n			1	2	1

Values tabled are means and, where available, standard deviations of K_{eq} determinations carried out as described in text. *n* = number of determinations.

At lower pHs and warmer temperatures (30–40°), our values of K_{eq} agree closely with those of other authors, collected by DYSON and NOLTMANN (1968). No earlier data include error estimates, but values of 0.30—0.34 at 30° and 40° are within the error envelopes of present data. There is discrepancy at 10°, where DYSON and NOLTMANN found values smaller than those at higher temperature, opposite to our results. These authors determined K_{eq} as stoichiometric balance between F6P and G6P at equilibrium. Redetermination of K_{eq} by this technique at low temperature gives values for K_{eq} of 0.833 ± 0.030 at pH 8, 10° (n = 8; equilibrium approached from four trials each of 100% G6P and 100% F6P), and of 0.577 ± 0.023 at pH 8.75, 10° (n = 6; three each approaches from 100% G6P and 100% F6P). There is close agreement with the Haldane equation results at pH 8 and only minor difference at pH 8.75. Preliminary studies suggest that part of the discrepancy between present results and those of earlier workers may be due to differences in ionic composition of working solutions—notably, the physiologically realistic presence of Mg^{2+} in all our solutions.

Within-genotype variation of K_{eq} estimates spans the whole range of *between*-genotype values, which is itself small for each set of conditions (Table 6). Such agreement is most encouraging as to the consistency of the data base.

Given the more extreme genotypic differences in K_m for G6P as compared with F6P, and the Haldane equation constraint, previously found patterns of genotypic difference in gluconeogenic V_{max} are not repeated in the glycolytic direction (Tables 7 and 8). Notably, *3/4* is not heterotic for glycolytic V_{max} as it is for gluconeogenic V_{max}. Other genotypes are heterotic for glycolytic V_{max} under particular conditions, e.g., *2/3* at 30°, pH 8.75. In general, gluconeogenic V_{max} diminishes with lowering pH, but glycolytic V_{max} first rises and then falls as pH decreases. Q_{10}s vary with both specific temperature range and genotype and are not the same in the two directions. These trends, other than the genotype-specific variation, approximately follow trends of K_{eq} change with reaction conditions.

We can now answer decisively the question, raised earlier (WATT 1977), whether these genotype-specific differences in V_{max} are due to differences in the actual catalytic rate constants k_{cat}, or whether differences in enzyme concentration ([E]) among genotypes, due to differential enzyme synthesis, might be responsible. Both in the V_{max} ratio as tabled here and in the left side of the Haldane equation (2), *[E] cancels out.* This converts the V_{max} ratio to the ratio of k_{cat}s in the two reaction directions. For all genotypes examined, the substitution of experimentally determined K_m values, gluconeogenic V_{max} values and $V_{max} = k_{cat}$ ratios for a given pH and temperature into the Haldane equation results in close agreement among genotypes in the values of K_{eq} generated, as observed. Thus, the genotype-specific V_{max} values must owe their differences entirely to differences in k_{cat}.

V_{max}/K_m ratios: Data for all genotypes at pH 8.75 appear in Table 9, data for the three most common genotypes at lower pH appear in Table 10, and the behavior of the V_{max}/K_m ratio in the glycolytic direction for the most common genotypes under all conditions is illustrated in Figure 2. The proportionality of the V_{max}/K_m ratio between the two reaction directions, at any given pH and

TABLE 7

Colias PGI V_{max} and V_{max} ratio values at pH 8.75

		Genotypes									
		2/2	*2/3*	*3/3*	*3/4*	*4/4*	*4/5*	*5/5*	*2/4*	*2/5*	*3/5*
10°	G→F	0.0181	0.0126	0.0141	0.0159	0.0224	0.0178	0.0170	0.0077	0.0164	0.0087
	F→G	0.0199	0.0163	0.0134	0.0180	0.0141	0.0159	0.0168	0.0138	0.0180	0.0154
	Ratio	0.911	0.776	1.053	0.882	1.592	1.119	1.009	0.556	0.912	0.568
			±0.015	±0.102	±0.078						
	n	1	3	2	2	C	C	C	C	C	C
30°	G→F	0.0435	0.0528	0.0346	0.0327	0.0387	0.0494	0.0458	0.0384	0.0433	0.0277
	F→G	0.0933	0.0816	0.0657	0.0816	0.0657	0.0816	0.0848	0.0753	0.0827	0.0774
	Ratio	0.466	0.647	0.527	0.401	0.589	0.605	0.540	0.510	0.524	0.358
		±0.008	±0.004	±0.014	±0.008	±0.006					
	n	3	3	6	2	4	C	C	C	C	C
40°	G→F	0.0960	0.0650	0.0520	0.0409	0.0593	0.0684	0.0732	0.0563	0.0483	0.0923
	F→G	0.1746	0.1368	0.1185	0.1383	0.1027	0.1346	0.1330	0.1072	0.1331	0.1304
	Ratio	0.550	0.475	0.439	0.296	0.577	0.508	0.550	0.526	0.363	0.708
				±0.016	±0.004	±0.014					
	n	1	1	4	2	4	C	C	C	C	C

V_{max} in EC units of activity/milligram of abdominal weight as justified in WATT (1977). C = ratio value calculated from the Haldane relationship, equation (2) of text. Other V_{max} ratios determined by experiment. Original V_{max} differences in the gluconeogenic direction at 30°, and V_{max} vs. temperature data from WATT (1977).

TABLE 8

V_{max} and V_{max} ratio values for Colias PGI genotypes at low pH

		Genotypes		
		3/3	*3/4*	*4/4*
pH 8.0				
10°	G→F	0.0181	0.0219	0.0306
	F→G	0.0180	0.0294	0.0187
	Ratio	1.003 ± 0.021	0.744 ± 0.014	1.638 ± 0.108
	n	2	2	2
30°	G→F	0.0384	0.0426	0.0407
	F→G	0.0620	0.0770	0.0620
	Ratio	0.619 ± 0.062	0.553 ± 0.027	0.656 ± 0.082
	n	3	6	5
40°	G→F	0.0800	0.0760	0.1181
	F→G	0.0913	0.1236	0.1227
	Ratio	0.876 ± 0.003	0.615 ± 0.024	0.963 ± 0.092
	n	2	2	2
pH 7.25				
30°	G→F	0.0395	0.0262	0.0296
	F→G	0.0753	0.0765	0.0582
	Ratio	0.525	0.342 ± 0.029	0.508
	n	1	2	1

Units as in Table 4.

TABLE 9

Colias PGI V_{max}/K_m ratios at pH 8.75

		Genotypes									
		2/2	*2/3*	*3/3*	*3/4*	*4/4*	*4/5*	*5/5*	*2/4*	*2/5*	*3/5*
10°	G→F	1.11	0.98	0.83	1.16	0.79	0.81	0.90	1.05	0.94	1.02
	F→G	1.60	1.41	1.20	1.64	1.14	1.16	1.29	1.52	1.36	1.47
30°	G→F	3.06	2.45	1.87	2.62	1.83	2.38	2.81	2.49	2.55	2.20
	F→G	14.14	11.33	8.65	12.0	8.65	11.03	13.05	11.58	11.81	10.18
40°	G→F	3.00	2.14	1.71	2.37	1.70	2.11	2.20	1.92	1.99	2.16
	F→G	15.32	10.94	8.77	12.13	8.70	10.77	11.27	9.83	10.16	11.05

Units are in V_{max} (Table 4) × $10^4/K_m$, as in WATT (1977).

temperature, is invariant across genotypes, as it is simply the value of K_{eq} in the Haldane equation (2). Values of V_{max}/K_m are highly specific to genotype, temperature and pH. Comparing Tables 6 and 7 with the earlier data, it seems that, in Colias PGI, genotypic variation in K_m is more effective in controlling variation of the V_{max}/K_m ratio than is variation in V_{max}.

Because of the fixed proportionality between reaction directions, the variation of the V_{max}/K_m ratio with genotype presents the same pattern in both reaction directions. That is, *3/4* is heterotic at all temperatures, *3/5* is heterotic at low temperatures, and otherwise heterozygote intermediacy with partial dominance

TABLE 10

Colias PGI V_{max}/K_m ratios at low pH

		Genotypes		
		3/3	3/4	4/4
pH 8.0				
10°	G→F	1.04	2.05	1.05
	F→G	1.19	2.35	1.20
30°	G→F	2.00	3.74	2.14
	F→G	5.74	10.74	6.15
40°	G→F	2.94	5.17	3.99
	F→G	9.80	15.68	11.80
pH 7.25				
30°	G→F	2.32	2.49	1.58
	F→G	6.60	7.08	4.51

Units are as in Table 6.

is the rule. Sometimes a higher V_{max}/K_m ratio is more dominant (e.g., 2/4 at 10°), sometimes the lower ratio is dominant (e.g., 4/5 at 10°). Indeed, dominance can shift with temperature: 4/5 is more like 4/4 at 10° and more like 5/5 at 40°. V_{max}/K_m ratios fall off from high temperature to low for all genotypes, but heterozygotes in general (4/5 excepted) differ less across temperatures than do homozygotes.

The V_{max}/K_m ratio decreases, in general, for the gluconeogenic direction with fall in pH but shows a maximum at pH 8.0 in the glycolytic direction, due to a faster drop with pH in K_m than in V_{max}. 3/4 remains heterotic under all conditions, and the previously noted tendency to asymmetry of heterosis for this genotype persists or intensifies with lowered pH, 3/3 becoming much closer to the superior heterozygote than is 4/4, especially at 30° at pH 7.25.

Interaction of V_{max}/K_m with previous findings on thermal stability: The data base on genotypic differences in k_{cat}, hence V_{max} among Colias PGI genotypes, is (properly) based on fresh, newly eclosed adults. However, there are drastic differences among PGI genotypes in sensitivity to environmental thermal stress (WATT 1977). Thus, the [E] component of V_{max}, in thermally sensitive genotypes, can be reduced by an individual's experience of heat stress. Figure 3 illustrates this, drawing data on thermal stability from WATT (1977) and on low temperature V_{max}/K_m in the glycolytic direction from Table 9. For example, a fresh animal of 2/2 PGI genotype would have a major advantage of V_{max}/K_m over a fresh 4/4. But, with one-fourth the thermal stability of 4/4, a 2/2 might, after some days in an environment with high daily maximum temperatures, have suffered sufficient thermal denaturation to have lost much or all of its original kinetic advantage. Similarly, although the thermal stabilities of these genotypes are much closer, the kinetic similarity of 3/3 to 3/4, and both their kinetic advantages over 4/4, might be gradually diminished with individual age in a high heat stress environment, given their relative stabilities: $3/3 < 3/4 < 4/4$.

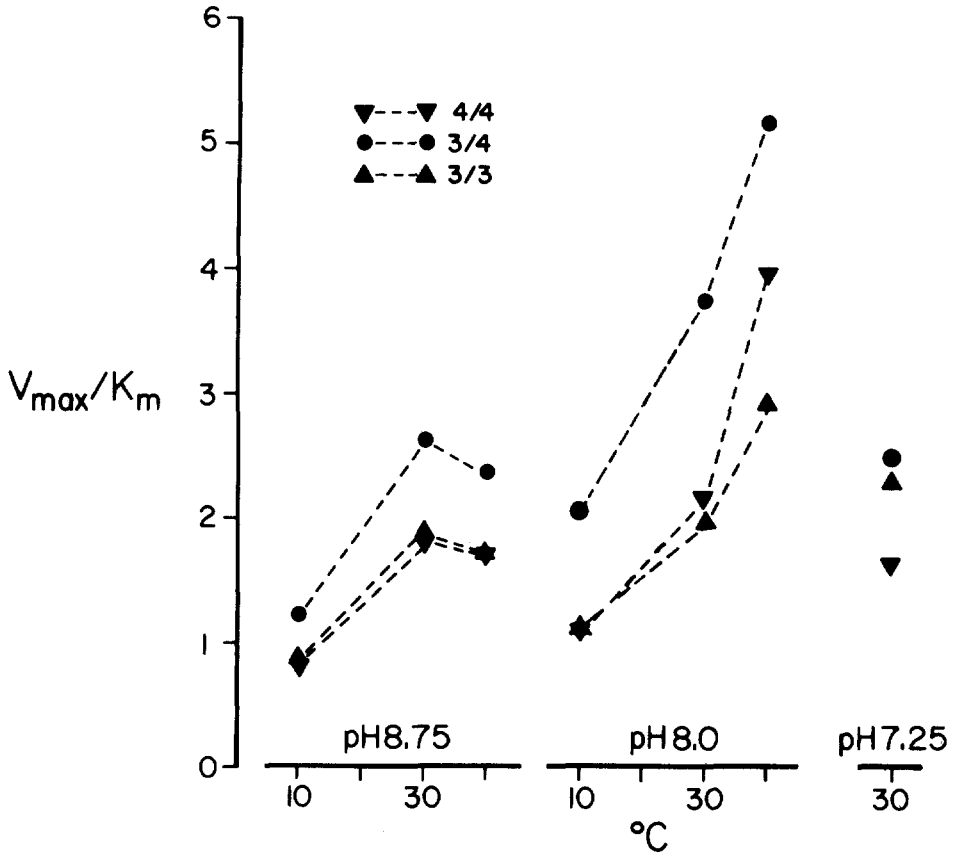

FIGURE 2.—V_{max}/K_m ratios in the glycolytic direction for the three most common Colias PGI genotypes. Units are as given in Table 6.

Such effects would be gradually mitigated after heat stress episodes if there is resynthesis of PGI during adulthood in Colias, although they would always be felt to some degree before completion of such resynthesis. Future work will examine this question of resynthesis explicitly.

PREDICTIONS

We can now predict, from biochemical data in hand, what fitness-related properties insects of the several genotypes might display in the wild. Thermal aspects of Colias' niche, especially the thermal distribution and dependence of flight activity, are important here as sources of potential selective pressure. Colias' thermal ecology, summarized in APPENDIX II, gives rise to two kinds of selective pressure: those that are involved with direct threats to individual survival and those that relate to fecundity components of fitness, especially through time available for flight activity.

Threats to survival may be divided into risks of predation and risks of physical damage from weather.

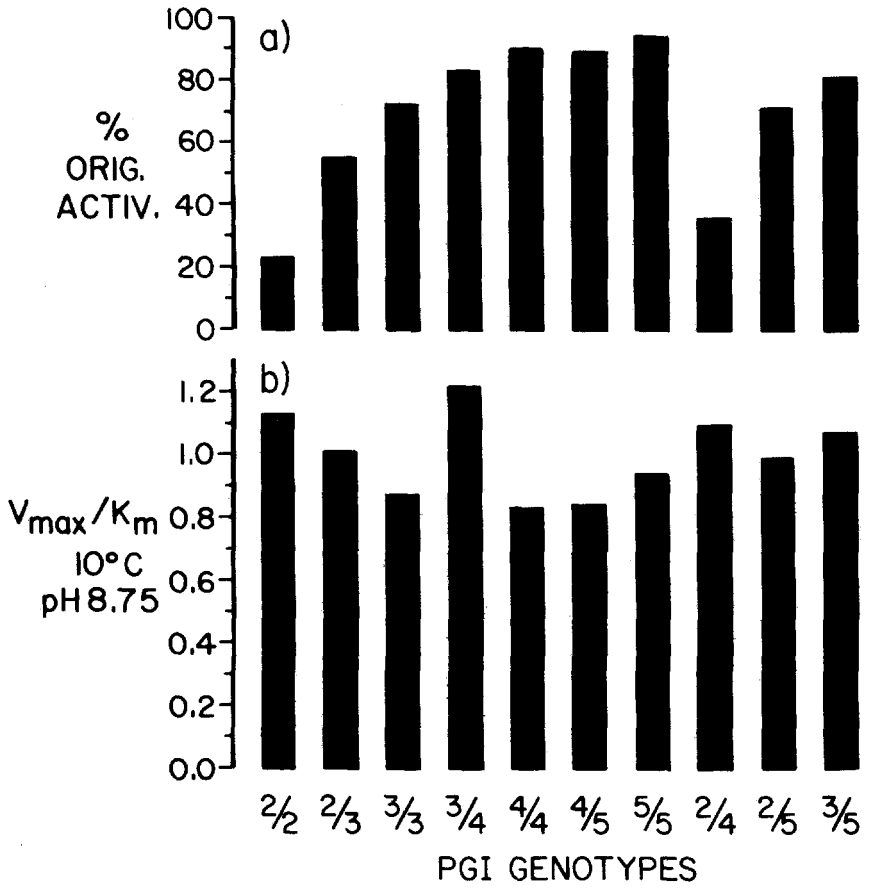

FIGURE 3.—Comparison of thermal stability and low temperature kinetic function in the glycolytic direction for Colias PGI genotypes. a) mean % activity remaining in purified preparations after standard heat shock at 50°; data from WATT (1977). For details and statistical validation of differences ($P \ll 0.001$), consult that reference. b) V_{max}/K_m ratios from Tables 6 and 7 of this paper; units as given in Table 6.

Predation by birds or small mammals on Colias appears to occur primarily when the animals are outside the nightly roost, but not yet warm enough to fly away from an attack (URBINA 1976; E. M. GONTERO and W. B. WATT, unpublished observations). Once Colias adults are at their thermal optimum, and, therefore, are fully mobile, such attacks do not appear to succeed often. But, the ability of glycolysis to support Colias' escape activity at a suboptimal temperature may be a limiting characteristic of crucial selective value.

Invertebrate, usually "sit-and-wait" predators, such as spiders concealed in the foliage of nectar-source or oviposition plants, may be the major cause of predation mortality on Colias at flight temperatures. There may be a premium here on quick flight response to avoid the lunge of such a predator, or on quick agitation to struggle free, and fast glycolytic flux response may be important to this.

The onset of storms (frequent at middle to high altitudes) brings a sudden clouding over and a drastic drop in solar load, hence a sudden drop in average Colias body temperature (T_b) which may presage hard rain or even hail by only a few minutes. This places a premium on maintaining flight maneuverability while cooling, so as to find suitable shelter from precipitation, whose direct impact may be fatal or seriously damaging to such small insects.

Flight activity time is a major index of fitness for Colias, since the insects must fly to feed, to find and court mates, to find suitable host plants and lay eggs, as well as to escape predators or disperse for other reasons. WATT (1968) first pointed this out qualitatively, and KINGSOLVER (1983b) has begun the quantitative documentation of the idea. Any characteristic that broadens the time envelope available for flight during the day, by allowing flight under suboptimal thermal conditions, etc., will contribute to physiological components of survivorship and of reproductive output by facilitating feeding and will contribute directly to reproduction by facilitating mating and oviposition. Beginning flight earlier in the cold morning, being able to continue it more effectively into cold, partly cloudly weather, being able to be more active under overheating conditions, or simply having longer endurance through the day would all broaden flight activity time. Variation in all such traits would be material for the action of thermally based selection pressures.

We now predict as follows, drawing both on biochemical and thermal-ecological information:

A. Overall biogeography of allele frequencies

1. Because of greater kinetic effectiveness, especially at low temperature, genotype combinations involving alleles *2* and *3* will be most favored in cold habitat populations.

2. Because of increasing thermal stability of genotype combinations among alleles *2, 3, 4* and *5* in that order, populations in warmer habitats, with higher chance of overheating stress, should show progressively higher frequencies of the higher numbered alleles, except that:

3. With small advantage over allele *4* in thermal stability, and poor kinetics of certain heterozygotes, especially *4/5*, allele *5* will not increase dramatically in frequency, even in the warmest environments, as compared with allele *4*.

4. If long-term directional change in average habitat temperature occurs, Colias PGI allele frequencies should track such change, alleles *2* and *3* increasing in cooling trends, alleles *4* and *5* increasing in warming trends.

B. Genotype survivorship over single generations

1. In environments with low to moderate ambient temperature, especially with cold nights, kinetic advantages among genotypes will be paramount. Hence,

a. *3/4* will show better survivorship than *3/3* or *4/4*, due to its heterosis for glycolytic flux capacity and response time, hence greater ability to avoid meteorological or other physical hazards and predators; *3/3* will be less disadvantaged than *4/4* in this respect.

b. In single-cohort populations with *3* and *4* as most common alleles, this will lead to major accumulation of heterozygote excess over the generation's flight period.

c. Of the two most common homozygotes, 4/4 will in general be more deficient than 3/3.

d. Overheating stress, to the extent present, will diminish the enyzme concentration component of V_{max}/K_m for the least stable genotypes, particularly 2/2 and 2/4, thus impairing these genotypes' survivorship.

e. 4/5 will be kinetically disadvantaged; 5/5 will be disadvantaged under the coldest conditions.

f. The other heterozygotes may be advantaged in survivorship to some degree, especially 3/5 at low temperature. Thus, these may also make some contribution to accumulation of overall heterozygote excess.

2. In warmer environments, as the extent of overheating increases, kinetic differences will interact with differences in thermal stability, the V_{max}/K_m ratios of less stable genotypes declining to the extent that thermal denaturation is not compensated by (hypothetical) enzyme resynthesis. Hence.

a. 3/3 will show poorer survivorship than in colder conditions, as to some extent will 3/4; 4/4 will survive relatively better, as it will retain more of its starting V_{max}/K_m value. There will be less or no accumulation of heterozygote excess due to 3/4 survivorship. In *extreme* heat stress, 4/4 may survive longer than 3/3 or 3/4.

b. 4/5 and 5/5 will aso survive better due to their thermal stability. 2/2, 2/3 and the minor heterozygotes will be disadvantaged in survivorship compared to them.

C. Genotype fecundity—flight time component

In general, as their V_{max}/K_m ratios are progressively higher, genotypes should be able to begin suboptimal flight under colder morning conditions, fly over a broader span of the day and maintain higher activity levels under marginal weather conditions. Hence,

1. Among the genotypes most common in the lowland species complex, 3/4 should begin flight soonest each morning and fly over the broadest span of time per day, with 3/3 being close to it in performance and 4/4 being much poorer.

2. 4/4's disadvantage in flight time should be decreased by previous episodes of heat stress, reducing the kinetic advantage of 3/4 through its lesser thermal stability; the advantage of 3/3 over 4/4 should be even further reduced by the same means.

3. Other genotypes should also show variation in flight onset and breadth of daily flight time in the same direct proportionality to their net V_{max}/K_m ratios, as controlled by interaction of intrinsic kinetic differences, thermal stabilities and environmental experience of thermal stress.

4. There should, thus, be disproportionate flight of heterozygotes early in the day or under cold weather conditions as compared with temperatures allowing peak flight density; whether disproportionate flight of heterozygotes occurs late in the day may depend on interaction of thermal conditions with the insects' own activity rhythms, which can affect time of roosting (E. M. GONTERO and W. B. WATT, unpublished results).

D. Genotype fecundity—gametogenesis component

Metabolic flux through glycolysis toward gametogenesis should be affected by any kinetic limitations at the PGI step. However, evidence is not yet on hand

to show just how much the rate of gametogenesis would be affected by given degrees of change in glycolytic flux. Thus, it is not yet clear to what extent PGI genotype substitution effects on gametogenesis would be detectable.

Check of predictions: Facts in agreement with predictions A1–A3 were reported by WATT (1977). The accumulation of heterozygote excess with age in single-cohort populations under low or moderate temperature, predictions B1b and B1e, was also reported at that time. The effect has continued to be apparent since (*e.g.*, Table 1, sample 4). Greater heterozygote frequency in flight under marginally cold weather, as compared with optimal, conditions, part of prediction C4, has been reported here, in RESULTS.

Prediction B1c can be checked against data already on hand but not analyzed in that way. The prediction applies to the lowland species complex, specifically to *C. p. eriphyle* and *C. eurytheme*. Among samples reported by WATT (1977) and Table 1, six are from single-cohort generations flying under low to moderate temperatures, satisfying the conditions of prediction B1, and are late enough in the flight season to show heterozygote excess > 0.05 of the sample. These have been analyzed with Wilcoxon's matched pair signed rank test. In all cases but one, *3/3* is less deficient below Hardy-Weinberg expected frequency than *4/4*, as predicted. That one exception is the lowest ranking difference among the pairs. A one-tailed test is proper, since a specific directional difference is predicted and a "significant" deviation in the other direction would be no more or less a violation than a mere absence of trend. $T_s = 1$, $n = 6$, and $P = 0.031$; the prediction is confirmed.

An accompanying paper (WATT, CASSIN and SWAN 1983) reports the test and verification, in natural Colias populations, of predictions concerning PGI genotypes' survivorship under extreme heat stress, the overall PGI heterozygosity increase among those flying early in the day *vs.* peak flight density, and the timing and breadth of flight activity by the three most common PGI genotypes.

DISCUSSION

CAVENER and CLEGG (1981) have properly emphasized the need for caution in extrapolating allozyme kinetic differences from particular *in vitro* conditions to general *in vivo* applicability. Glycolytic polymorphisms were chosen for study in this work to minimize the inherent difficulties of *in vitro-in vivo* comparison, by the very cytoplasmic and soluble nature of the enzymes of this pathway itself (WATT 1977; *cf.* GARFINKEL and HESS 1964, HESS 1973). In light of this, the continued congruence of our predictions from biochemical data with field observations is most encouraging, suggesting that, for Colias PGI at least, *in vitro* study sheds effective light on events *in vivo*. Further successful test of *de novo* predictions, reported next (WATT, CASSIN and SWAN 1983), provides more support for this view.

It is not really surprising that negative covariation or none has been found among Colias' PGI alleles. There is, even among taxa distantly related in the genus, a pattern of two common alleles ($0.2 < p_i < 0.7$) making up most of the allele frequency, two frequent alleles ($0.01 < p_i < 0.2$) and one to three rare alleles ($p_i < 0.01$). The minimum sum of $p_3 + p_4$ seen in *C. p. eriphyle* is 0.80,

and the average is 0.89, whereas in *C. meadii* the minimum of $p_2 + p_3$ seen is 0.93 and the average is 0.95. These allele frequency statistics behave basically as *two*-allele polymorphisms, "invaded" to varying degrees by the frequent alleles, whereas the rare alleles are simply rare. This pattern extends to *C. alexandra* Edwards and *C. eurytheme*, even though among all these taxa the identity of the alleles in common, frequent and rare classes varies with habitat temperature in relation to thermal stress stability of the different genotypes (WATT 1977).

These findings have eliminated the possibility (alternative 3) that population-structure factors have operated on a selectively neutral polymorphism to generate heterozygote excesses. They reinforce the view that natural selection acts directly on this locus and diminish sharply the credibility of alternative 2, that "hitchhiking" with some unknown linked locus is responsible for the PGI genotypes' field behavior, in spite of the congruence between genotypes' functional characteristics their variation patterns in the field. The system now appears as if a basically directional, overall thermal selective regime were setting "boundary conditions" for the occurrence of alleles within taxa and combinations of selective pressures in space and time were maintaining variability within those boundaries. In the lowland species complex, the primary mechanism for maintaining variation is the direct heterosis, in kinetic function, of the most common heterozygote 3/4. It appears from present data that the "frequent" alleles in this case are maintained by combinations of fluctuating environmental pressures acting on opposed trends of variation in stability and in kinetics among the other genotypes. Environmental fluctuation may be the primary mechanism maintaining variability at this locus in the alpine *C. meadii*, because in that species truly heterotic genotypes are rare. It is noteworthy that in *no* case are heterozygotes exactly intermediate in properties between their homozygotes, nor are there any other manifestations of simple additivity among the genotypes at this locus.

KACSER and BURNS (1981), applying their analytic viewpoint on *in vivo* pathway organization to the long-standing problem of dominance, have made remarks in passing that require comment in the present context. Although they point out that reactions close in the "metabolic map" to a given reaction will have larger sensitivity coefficients with respect to that step, compared with more distant reactions, these authors also stress a conclusion that "most variation has small or negligible effects." Casual misreading of this statement and its context can lead to the impression that KACSER and BURNS are arguing against one of their own major conclusions: that all steps in a pathway will have some effect upon the kinetic performance of that pathway, unless they are mutually adjusted to centralize such effects in one or a few steps. Were such an impression correct, not only should the present findings be unimportant, but CAVENER and CLEGG (1981) should not have found major physiological differences, which they did, among allozymes of Drosophila's pentose shunt. It is, of course, incorrect.

KACSER and BURNS have demonstrated what, as they acknowledge, WRIGHT (1934) argued in earlier terms: dominance may frequently be the result of kinetic

organization among individual steps of pathways, whether explicitly selected or a consequence of other organizational features. If flux through a pathway has low to intermediate sensitivity to one of its reaction steps, then a heterozygote for alteration of that step may have only a minor effect on pathway flux. If the pathway in question is remote from central mass-energy processing, and its products, however qualitatively essential, of minor quantitative impact on the organism's whole mass-energy budget, it may be that heterozygosity for such effects will be indistinguishable at the gross phenotypic level among much other similar variation elsewhere in the genome and the metabolic map.

However, it does *not* follow from this, nor do KACSER and BURNS argue, that enzyme kinetic variation in central pathways of mass-energy processing should be undetectable in effect. KACSER'S research group (BARTHELMESS, CURTIS and KACSER 1964) has shown in the Neurospora arginine pathway that, as their analysis would predict, kinetic changes at individual steps do entail major changes in flux levels, increased pool sizes of metabolic intermediates and so forth. The pentose shunt and glycolysis constitute the major flow of carbon mass from food inward to metabolism, whether to pentose synthesis, storage as glycogen *or* fat or flux onward to the Krebs cycle and, thus, potentially on into amino acid biosynthesis. Between them they exercise primary influence on both redox balance and energy charge in the cell. Glycolysis is central to all ATP-based energy supply, specifically so in animal muscle. KACSER and BURNS' analysis provides some justification for expecting a gradient of selection pressure across the metabolic map—but we should expect glycolytic loci such as PGI, and the pentose shunt loci studied by CAVENER and CLEGG, to be near the high end of that gradient.

Why should the PGI locus be a focus of intense natural selection? One possible answer is this: if allosterically modulatable steps are actually to control flux through their pathways, the intervening steps must be selected to have low sensitivities (KACSER and BURNS 1973, 1979; see previously and APPENDIX I) with respect to the control steps and, hence, to system flux as a whole. Now, the PGI step occurs just prior to the phosphofructokinase control step in glycolysis. In particular, in Colias flight muscle, which experiences dramatic transients in glycolytic flux demand caused by flight activity, that demand is supplied both by flux from hemolymph trehalose through trehalase and hexokinase steps to G6P (C. L. BOGGS, W. B. WATT, and J. M. SCHMITT, unpublished data) and by flux from local glycogen storage into the G6P pool, then into the PGI step. The PGI step must thus be selected to minimize its sensitivity and its transient time with specific respect to the phosphofructokinase step, while receiving input from two sources of G6P which will have very different transient behavior *and* steady state flux capacities. A full quantitative assessment will become possible as our study of glycolytic polymorphisms *in vivo* advances.

What is there is about the protein structure of Colias PGI that leads to such major functional differences among its genetic variants, which then interact powerfully with selective pressures? The question is pertinent both to primary structural differences in amino acid sequence and to the resulting tertiary-quaternary structural differences which lead to the observed differences in

kinetics and thermal stability. There is an obvious expression of interallelic complementation (*cf.* FINCHAM 1966) in the observed heterosis for kinetics—under all conditions in the *3/4* genotype, for most heterozygotes at low temperature. Structural understanding of this may be of special interest.

HOCHACHKA and SOMERO (1973) suggested that adaptive adjustment of enzyme functional characteristics might frequently have to "trade off" kinetic flexibility and effectiveness, particularly at low temperature, against stability to high temperature stresses. This implies that sharp limits may exist on the total degrees of freedom available, *i.e.*, the number and nature of alternative functional configurations possible, in proteins. It contrasts with facile assumptions often made, either that proteins tolerate a wide variety of substitutional changes without major functional effect or that adaptive adjustments of protein structure can be made in whatever functional direction is "optimal," without regard for constraints arising out of the preexistent structure itself.

The present results support HOCHACHKA and SOMER'S idea remarkably well. Referring to Figure 3, the three heat-stable genotypes, *4/4*, *4/5* and *5/5*, are three of the four least kinetically effective genotypes at low temperature. Moreover, the three least heat-stable genotypes, *2/2*, *2/3* and *2/4*, are all kinetically effective at low temperature. The only exceptions to an inverse correlation of kinetic effectiveness with thermal stability are the two heterozygotes *3/4* and *3/5*: both are thermally stable although not maximally so, but, whereas *3/4* is kinetically heterotic throughout conditions studied, *3/5* is so only at low temperature (Tables 1 and 6). In this system there is no one functionally best genotype in all respects and in particular there is no optimal homozygote. Even the *3/4* heterozygote, which combines the best kinetic phenotype found with a *near*-maximal thermal stability, shows some constraint on simultaneous adjustment of these properties. This picture of biochemical adaptation to environmental pressures, but only within sharp constraints, is quite consistent with the recent reminder by GOULD and LEWONTIN (1979) that this is not the best of all possible adaptive worlds. Preexisting structure may constrain possibilities of adaptation quite as much at the enzyme mechanism level as at the developmental program level.

With respect to these structural and functional questions, it may be uniquely informative to study those alleles of Colias PGI that occur in the wild but are rare, having frequencies of 0.005 or less. Two alleles, *1* and *6*, turn up with some regularity; the third, allele *7*, has been seen but once (see previously). It now seems that there are adaptive reasons for the evolutionary success of the four frequent-to-common alleles. We can then ask why additional alleles are not favored. This may prove to be a generally useful property of multiallele polymorphisms, if they are selected upon at all: it may be possible to study true gradation of favorable to unfavorable selective pressure among the members of such polymorphisms.

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APPENDIX I

BIOCHEMICAL CORRELATES OF FITNESS

Evolutionary criteria of kinetic function at the PGI locus: The interconversion of G6P and F6P, catalyzed by PGI, is, as enzyme-catalyzed reactions go, a simple one. The simplest realistic reaction mechanism would be:

$$G6P + PGI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} G6P - PGI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} F6P - PGI \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} F6P + PGI$$

DYSON and NOLTMANN (1968) proposed a detailed reaction mechanism with four "interior" complexes rather than two, but since this is a unisubstrate reaction in each direction, the overall initial velocity kinetics would be the same (PLOWMAN 1972; SEGEL 1975). There is for either case the same reversible Michaelis-Menten, or Briggs-Haldane, net velocity (V_{net}) equation. If the glycolytic direction be taken as "forward," this equation would be:

$$V_{net} = \frac{\frac{V_{max_{G\rightarrow F}}}{K_{m_{G6P}}} \cdot [G6P] - \frac{V_{max_{F\rightarrow G}}}{K_{m_{F6P}}} \cdot [F6P]}{1 + \frac{[G6P]}{K_{m_{G6P}}} + \frac{[F6P]}{K_{m_{F6P}}}} \quad (1)$$

The identity of individual rate constants contributing to the composite constants V_{max} and K_m would differ, depending on reaction mechanism details. But, it does not follow from this, as some assert, that no straightforward functional meaning is assignable to the composite constants. V_{max} values still express reaction velocity at saturating reactant concentrations, and the K_m is still a relative index of substrate binding affinity (or conversely a measure of the ease of enzyme saturation by substrate), being numerically equal to the substrate concentration producing $v = V_{max}/2$ in the (initial) absence of the other substrate. Further details, and derivations, may be found in PLOWMAN (1972), SAVAGEAU (1976) and SEGEL (1975).

If both substrates are saturating, then net flux (v_{net}) in (1) reduces to the difference of V_{max} in the two directions. But, this is seldom if ever so for glycolytic enzymes (e.g., LOWRY and PASSONNEAU 1964; HOCHACHKA and SOMERO 1973; CORNISH-BOWDEN 1976). In the fly Phormia, SACKTOR and WORMSER-SHAVIT (1966) found F6P and G6P pool concentrations far below what would be needed to saturate Colias PGI. In rat brain, glycolytic $[S]/K_m$ ranges from 0.1, or 9% saturation, to 18, or 95% saturation, the latter for the enzyme aldolase which is operated *in vivo* counter to its natural equilibrium (LOWRY and PASSONNEAU 1964). In such cases, the net flux is the difference between products of V_{max}/K_m ratios with substrate concentrations for each reaction direction, adjusted by a denominator incorporating the degree of saturation, as $[S]/K_m$, in both directions (1). Thus, the ratio V_{max}/K_m is more directly expressive of net flux capacity than either single composite constant, although K_m still plays a separate role by setting the level of saturation and, thus, the distance from control of step flux by V_{max} alone. The *proportionality* of V_{max}/K_m ratios in the two reaction directions is fixed by the equilibrium constant for the reaction, as expressed in the appropriate Haldane equation (HALDANE 1930):

$$\frac{V_{max_{G\rightarrow F}} K_{m_{F6P}}}{V_{max_{F\rightarrow G}} K_{m_{G6P}}} = K_{eq} \quad (2)$$

The substrate concentrations themselves are *variables dependent upon* the kinetic parameters of the PGI step and the rest of the glycolytic pathway (EASTERBY 1973; KACSER and BURNS 1973, 1979). Changes among PGI genotypes in kinetic parameters will alter the relevant substrate (and product) concentrations. For example, a PGI genotype showing a larger V_{max} and/or smaller K_m, hence a larger V_{max}/K_m ratio, than another will, as a result, maintain a lower substrate concentration, in transient or steady state, than will that other genotype, all else being equal (EASTERBY 1973; KACSER and BURNS 1973, 1979).

The V_{max}/K_m ratio is the inverse of the time constant for the reaction step in a transient state (EASTERBY 1973, HESS 1973). A large value of this ratio is, thus, important to the responsiveness of the PGI step and of glycolysis as a whole, since the transient time of the pathway is the sum of all step transients. Colias glycolysis, like that of other insects, must respond quickly to changes in flux demand caused by flight onset.

A similar importance of the V_{max}/K_m ratio to steady state flux capacity is seen from the analysis of KACSER and BURNS (1973, 1979). They consider how flux through any one step in a pathway, which in the steady state is equal to the whole-pathway flux, is affected by changes in kinetics not only of that step's enzyme, but of other enzyme-catalyzed steps in the pathway. These relations they term sensitivities and symbolize by Z_i, as the sensitivities of pathway flux to changes in steps $i = 1$ to n, the number of steps in the pathway. These Z_i are distributed among the different steps

in inverse proportion to V_{max}/K_m ratios (equilibrium constants for those steps also appear as multipliers). The sensitivities Z_i of pathway flux to all steps must sum to 1.

Now, an allosterically modulated step, such as phosphofructokinase in glycolysis, has ostensibly been evolved to exert major control over pathway flux. In KACSER and BURNS' analytical language, this locus has evolved a high controllability by certain metabolites, in this case the balance of energy charge (ATKINSON 1977). The whole-pathway flux is very closely approximated by the flux through this step under a wide range of conditions (e.g., GARFINKEL and HESS 1964). But this also requires the condition, in KACSER and BURNS' language, that the pathway flux's sensitivity to the control step is very close to 1 and its sensitivities to other steps are very small. In turn, therefore, the other steps must have evolved high V_{max}/K_m ratios, so that their Z values for system flux are very small. These other steps will, thus, appear on casual inspection to have "excess" flux capacity. Among other consequences, this reveals the error in the common assertion that only the control steps in a pathway are of evolutionary importance. Actually, these and the other steps must be coadapted with suitably high and low flux sensitivities, respectively, for adaptively useful allosteric control of the pathway to be possible.

Colias' PGI is a reaction step surrounded by several allosterically modulated enzymes—glycogen phosphorylase and synthetase, phosphofructokinase, in fat body trehalose-6-phosphate synthetase (MURPHY and WYATT 1965)—whose responses to energy charge (ATKINSON 1977) and related metabolic signatures (QUASTLER 1964) allocate carbohydrate resources to storage or to metabolic use (SACKTOR 1975). Thus, the V_{max}/K_m ratio should be a direct (but *not* necessarily linear) index of genotypic fitness at this locus. Large values of this ratio will reduce the PGI step's time constant for flux change under transient conditions and also will reduce its sensitivity value in relation to steady state glycolytic flux. This is selection to *avoid* inappropriate "control" of flux by the PGI step, allowing allosteric enzymes to exercise effective control over pathway function.

Small K_m will also be a positive fitness criterion. Small K_m means that lower levels of substrate concentration are needed for a given degree of substrate saturation; thus, K_m scales the consequences of genotypic change in the V_{max}/K_m ratio. Large V_{max} will be considered, but in context of its contribution to change in the V_{max}/K_m ratio, and also in context of the Haldane equation constraints (2).

FERSHT (1974) and CROWLEY (1975) have argued not only that the V_{max}/K_m ratio (or k_{cat}/K_m ratio, k_{cat} being the catalytic rate constant or turnover number) of an enzyme should evolve to be large, but that K_m should become "large relative to substrate concentration" (CROWLEY 1975). As CORNISH-BOWDEN (1976) points out, the latter effect is counter-intuitive and also does not seem to have occurred (*cf.* e.g., LOWRY and PASSONNEAU 1964). FERSHT and CROWLEY share assumptions of externally fixed substrate concentration and/or reaction velocity, with respect to which kinetic parameters evolve. But as already pointed out, fluxes and metabolite concentrations ordinarily vary within the individual on a short time scale, being variables dependent on genetically determined enzyme kinetic parameters (*cf.* KACSER and BURNS 1973, 1979, 1981) as well as acute metabolic demand. Thus, if a value of $[S]$ small with respect to K_m were adaptive, one would expect evolution of the kinetic parameters, assuming available genetic variability, to reduce $[S]$, rather than the evolution of poorer substrate binding by the enzyme in question. Circumstances, such as mass food digestion, may occur in which FERSHT and CROWLEY's conclusion may apply, but it appears irrelevant to the present case.

PLACE and POWERS (1979) have used as a fitness criterion the k_{cat}/K_m ratio, since this ratio renders comparisons independent of enzyme concentration. (Recall that $V_{max} = k_{cat}[E]$, $[E]$ = enzyme concentration.) Our data are in terms of saturated specific activity, V_{max}, as determined by averaging individual values for 11–20 fresh males of each genotype on the same randomly segregating genetic background. These V_{max} differences are actually k_{cat} differences, as shown before; direct estimation of k_{cat} values will follow once Colias PGI is purified to homogeneity. However, although PLACE and POWERS' usage is appropriate in many circumstances, we phrase the present discussion in terms of V_{max}/K_m ratios. Within V_{max}, $[E]$ is a parameter through which environmental challenges to PGI's stability can change kinetic differences among genotypes. Colias PGI genotypes differ greatly (WATT 1977) with respect to thermal stability. A heat shock can have a profound negative effect on $[E]$, of short or lasting duration depending on the degree of protein resynthesis and, thereby, on the V_{max}/K_m ratio of a thermally unstable genotype. Thus, placing our comparisons in terms of V_{max}/K_m allows discussion of the kinetic consequences of heat stress without change of context. In the longer

term, differences in k_{cat} and in [E] may well have different evolutionary importance, but that is beyond our present scope.

The pentose shunt metabolite 6-phosphogluconate (6PG) is an inhibitor of PGI (e.g., AVISE and KITTO 1973; PALUMBI *et al.* 1980; HOFFMANN 1981a). A possible physiological role for this interaction has been suggested (PALUMBI *et al.* 1980). Enzymes of the pentose shunt, e.g., glucose-6-phosphate dehydrogenase, cannot be detected in Colias flight muscle (W. B. WATT, unpublished results) and, thus, pentose shunt metabolites could not interact with Colias PGI in this tissue under acute flight activity demand. Thus, we have not yet studied effects of this metabolite on Colias PGI. As to interaction in fat body, where the pentose shunt does operate, the situation will need careful separate study. In some animal tissues, two pools of glucose-6-phosphate have been found, raising the possibility that the pentose shunt and glycolysis are compartmentalized away from one another, either within single cells or by specialization of distinct cell types (PALUMBI *et al.* 1980).

The in vitro-in vivo transition and appropriate study conditions: Solution conditions *in vivo*, such as pH, etc., might not be the same as those used in *in vitro* assays. If *in vitro* assays are done over a suitably broad range of solution conditions, however, the *in vivo* conditions will be bracketed. Next, the enzyme in question might interact with cellular ultrastructure *in vivo* so as to alter its kinetics. Avoidance of this problem was one of several major reasons for choosing glycolytic enzymes for evolutionary-genetic study (WATT 1977), as this pathway operates in solution in the cytosol. Glycolytic enzymes might form loose multienzyme complexes (e.g., MOSES 1968), giving rise to some degree of "substrate channeling," but no clear evidence for this exists in eukaryotes (in fact, some against it; DEDUVE 1972). In yeast or cultured mammalian cells, at least, if such effects are present, they do not disturb successful calculation of *in vivo* flux through glycolytic steps (specifically including PGI!) from kinetics constants determined *in vitro* (GARFINKEL and HESS 1964; HESS 1973).

Enzyme concentrations *in vivo* are higher than in typical *in vitro* assays. In yeast, glycolytic enzymes occur in concentrations of 10^{-4} to 10^{-5} M in the cytosol (HESS 1973), although this may be an extreme upper limit case. The effect is to reduce *free* substrate concentrations below the level of *total* substrate present, since so much substrate would be bound by enzyme. This would reduce effective enzyme saturation, emphasizing the importance of substrate binding as indexed by K_m and increasing the extent to which the V_{max}/K_m ratio, as opposed to V_{max} alone, governs realized flux. If this effect is accounted for in calculations, no conflict between *in vitro* and *in vivo* kinetics is to be expected—or is found in the cases noted before. This does not diminish the importance of carrying genotypic comparisons in the present system through to the *in vivo* level, as previously pointed out (WATT 1977). It does show that *in vitro* study can be informative as to what takes place *in vivo* and indeed can serve as a necessary "screening" process for the eventual feasible study of this varying system *in vivo*.

A case can be made that the glycolytic direction for the PGI reaction, converting G6P to F6P, may be more adaptively crucial than the previously examined gluconeogenic direction. The pH-stat assay procedure of DYSON and NOLTMANN (1965) has been adapted to this purpose. In addition to the previous assay temperatures of 10° and 30°, 40° has been added, at the upper end of the insects' thermal optimum. As to pH, preliminary results suggested that functional differences seen at pH 8.75, the gluconeogenic velocity optimum, persisted or actually enlarged at lower pH, so that this pH was conservative for evaluating such differences. However, most *in vivo* animal cytosol pH values fall between pH 6.7 and 7.5 (SOMERO 1978). Preliminary results (R. STEINHARDT and W. B. WATT, unpublished results) suggest that pertinent *in vivo* pH for Colias would at least be well below pH 8.75. Thus, exploration of genotypic differences in function at pH 8.0 and 7.25 has also been begun.

APPENDIX II

THERMAL ECOLOGY OF COLIAS AS SOURCE OF SELECTIVE PRESSURES

Adult Colias cohorts at the start of their flight periods always show PGI genotype arrays close to Hardy-Weinberg expectations, suggesting absence of major viability differences among genotypes in egg, larval or pupal stages. Such differences might still occur under special conditions, but for now we will be concerned with *adult* thermal ecology as a source of selective pressures on PGI. The

following is summarized, except as otherwise noted, from WATT 1968; TSUJI 1980; KINGSOLVER 1983a, b; KINGSOLVER and WATT 1983.

Colias are behavioral thermoregulators, basking laterally (*i.e.*, with wings closed above the body) perpendicular to sunlight when cold to maximize energy absorption, seeking shade or orienting parallel to sunlight to minimize absorpion when overheated. Adults of all Colias species display a common dependence of flight activity on body temperature (T_b). Maximum flight activity occurs in the range T_b = 35–39°. Little voluntary flight other than shade seeking occurs above this range, and flight diminishes in frequency below it to a limiting value of T_b = 29°, below which no voluntary flight occurs. Weak fluttering in escape from disturbance may be possible down to T_b = 25°. Males are more willing than females to fly in the suboptimal T_b = 29–35° range, especially under climatic or meteorological conditions such that mate-seeking time is limited. As grassland insects, Colias roost among plants at night, 1–10 cm above ground level (URBINA 1976; E. M. GONTERO and W. B. WATT, unpublished data) and in the morning must walk, at T_b far below values permitting flight, up into sunlight to bask for warm-up. Time available for activity at high T_b may affect the effectiveness of roost choice, especially if daily flight ends with sudden storm onset rather than gradual afternoon cooling.

Despite this uniform thermal physiology, Colias inhabit grasslands from warm temperate lowlands to cold alpine (or arctic) tundra and, hence, encounter very different regimes of air temperature, wind, solar load, degree of cloudiness, etc. They achieve flight in these different situations by adjustment (apparently by selection on controlling polygenes) of pigmentation, thus changing absorptivity for sunlight, and of fur distribution and thickness, thus changing insulation against convective heat loss. The limits of these adjustments in cold climates balance further gain in flight time, with yet darker color or thicker fur, against an increasing risk of physiologically unacceptable overheating in peak temperatures of the day, even in arctic or alpine habitats. In warm climates, converse limitations of possible adjustment occur. Thus, for example, cold-habitat populations will achieve optimum T_b at much lower air temperatures than warm-habitat populations.

Although Colias' T_b requirements are the same in all habitats, still an extreme cold-habitat population must withstand severe cold stress in moving from the roost to sunlight each morning, then must warm against low ambient temperatures, must maintain T_b suitable for activity in such ambient conditions, and still may suffer some overheating in occasional low wind speed, high solar load conditions. A warmer habitat population must warm from less stringent low temperatures but may be subjected to far more overheating, as in summer in the Central Valley of California, when *c. eurytheme* is forced to mid-day inactivity by overheating, flying at maximum density both before and after the daily temperature peak (LEIGH and SMITH 1959). Even in high avoidance behavior, animals in such conditions may experience T_b at least in the middle 40°s. There is a continuum, from populations with routine low temperature stress and occasional overheating stress, to populations with occasional low temperature stress and routine overheating problems.