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FLIGHT-MUSCLE ADENYLATE POOL RESPONSES TO FLIGHT DEMANDS AND THERMAL CONSTRAINTS IN INDIVIDUAL *COLIAS EURYTHEME* (LEPIDOPTERA, PIERIDAE)

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Summary

We study here the connections among body temperature variation, flight performance and flight 'fuel' metabolism in *Colias eurytheme* butterflies, to begin re-examining the metabolic reasons for animal thermoregulation. Methods are presented for (a) stable extraction of adenylates (and other metabolites) from the flight muscles of individual *Colias eurytheme*, (b) automated separation and quantitative analysis of individual adenylate samples by high-pressure liquid chromatography and (c) reliable, low-variance assay of inorganic phosphate levels in the same extracts. Correlations among metabolite concentrations and two indices of muscle cytosol ATP maintenance occur as expected on general metabolic principles. [ATP] and [ATP]/[ADP] decline from resting levels to reach a plateau in the first minute of free, interrupted flight, while [AMP] increases at the same time; these concentrations do not vary further for up to 6 min total flight time. In an initial test of the alternative metabolic bases of the thermoregulation of *Colias eurytheme*, we find that [ATP]/[ADP] rises between a body temperature, T_b, of 31 and 35 °C, at the base of the behavioral thermal optimum for flight, but then decreases again at T_b=39 °C, at the top of the behavioral thermal optimum and well short of damaging temperatures. This is not consistent with the view that metabolic effectiveness increases monotonically up to the lower limits of thermal damage to enzymes, but supports an alternative hypothesis that the narrowness of thermoregulation results from a system-based constraint on the breadth of temperature over which maximal energy processing is possible.

Key words: adenylate, high-performance liquid chromatography, individual metabolic performance, insect, flight, thermoregulation, butterfly, *Colias eurytheme*.

'Data on the biochemistry of insect thermoregulation are extremely scanty.'

G. A. Bartholomew (1987)

Introduction

Comparative physiologists and ecologists have studied insect flight and its dependence on insect body temperature for decades. The organismal energetics of insect flight have been studied by measuring gas exchange, and the thermal dependence of this has also been studied (e.g. Casey, 1981; Ellington et al., 1990). The thermal dependence of the biochemistry that supports flight (indeed, gives rise to organismal oxygen uptake patterns) has not been studied, although it is a prime example of metabolic regulation (Bailey, 1975; Sacktor, 1975). For example, Sacktor and Wormser-Shavitt (1966) and Sacktor and Hurlbut (1966) flew blowflies, *Phormia regina*, on a 'flight mill' for various times, freeze-clamped them with liquid nitrogen and quantified levels of glycolytic, Krebs cycle and adenylate metabolites using enzymic assays, finding that flight metabolism of *P. regina* does not reach steady state for many minutes after the onset of flight. Watt and Boggs (1987) obtained initial data on carbohydrate metabolism during flight in *Colias* spp. butterflies, Gmeinbauer and Crailsheim (1993) studied the flight metabolism of honeybees *Apis mellifera* and Wegener et al. (1991) used miniature nuclear magnetic resonance sensors to study arginine phosphate and ATP dynamics during locust flight. None of these studies, however, considered thermal aspects of flight metabolism.

This intersection of topics is central to the study of adaptation and constraint in evolutionary physiology and to the question of why animals thermoregulate. One common explanation is the 'maxithermy' hypothesis: because chemical reactions are thermally dependent, only at high body temperature (T_b) can metabolism support intense activity, so animals 'should' maximize T_b just short of the temperature at which their proteins are denatured. But, some animals achieve high metabolic rates at low T_b by increasing their concentrations of enzymes (e.g. Crawford and Powers, 1989), so alternative explanations are needed.

Bartholomew (1981) and Heinrich (1977, 1981) argue that

endothermy evolved in large animals because their metabolic intensity and low surface area/volume ratios constrain them to thermal excess during activity. Thus, large animals evolved to regulate T_b below thermal damage limits, but to values as high as possible for the sake of 'metabolic efficiency'. This explanation may well account for evolution of heat-loss devices in large insects (e.g. Heinrich, 1974). But, small- to medium-sized insects, whose low metabolic intensity and high surface area/volume ratio do not impose major overheating risk on them during activity, have also evolved regulation to high T_b and/or adherence to narrow thermal ranges when active at high or low T_b. Other causes must therefore also underlie thermoregulation in such insects (Watt, 1986, 1991). Biophysical thermal limits, e.g. on the elasticity of cuticle and muscle fibers, may contribute to this thermal specialization (e.g. Josephson, 1981). However, much of this specialization may also stem from the inability of metabolic pathways to exhibit high throughput and fine control over wide temperature ranges in insects or in other creatures (Watt, 1991). That is, thermoregulation may be a necessity constrained by the 'narrow thermal bandwidth' possible for the highly integrated function of metabolic pathways.

Colias spp. butterflies offer a fine system for testing these issues. They are laterally basking behavioral thermoregulators, orienting perpendicular to sunlight when cold, parallel to it when overheated and randomly when $35\,°C \leqslant T_b \leqslant 39\,°C$; flight occurrence and wingbeat frequency are also both maximized at T_b=35–39 °C (Watt, 1968, 1997; Kingsolver, 1983a; Tsuji et al., 1986). *Colias* spp. reach this genus-wide, common T_b optimum in thermally diverse habitats by genetic or photoperiodic adjustment of absorptivity for sunlight and insulation against convective cooling (Watt, 1968, 1969; Kingsolver, 1983a,b; Kingsolver and Watt, 1983). Nonetheless, they routinely experience wide, flight-limiting fluctuations in T_b. What are the metabolic effects of this fluctuation, and how do these effects contribute to flight limitation?

Like other forms of animal locomotion, insect flight is proximately fueled by transduction of ATP bond energy into muscle contraction. Drawdown of flight-muscle ATP at the start of insect flight is buffered (Stucki, 1980a,b) by the muscle phosphagen arginine phosphate and the adenylate kinase reaction $2ADP \leftrightarrow ATP+AMP$ (Sacktor, 1975). In *Colias* spp., as in many other insects, carbohydrate processing is essential to ATP replenishment beyond the first few seconds of flight (Bailey, 1975; Sacktor, 1975; Watt and Boggs, 1987). The study of flight-muscle ATP resupply in a thermal context is thus central to understanding how metabolic mechanisms contribute to the thermal dependence of flight and the need for thermoregulation.

Here, we present techniques for the quantitative analysis of adenylates and inorganic phosphate in extracts of single *Colias eurytheme* thoraces. We document patterns of variation in adenylate levels during voluntary flight. We then make a first-approximation test between 'classical' and 'narrow-bandwidth' explanations for the dependence of metabolic function on T_b. If the former is correct, metabolic support of flight-muscle [ATP] should increase in, or at least maintain, effectiveness right through the behavioral thermal optimum. If any version of the latter is correct, maintenance of [ATP] should peak and then fall again with rising T_b, well before thermal damage temperatures are reached.

Materials and methods

Experimental stocks, rearing procedures and genetic considerations

Our laboratory colony of *Colias eurytheme* produces one generation every 5 weeks. Vetch (*Vicia* sp.) is used as larval food. We maintain a wild genetic background in the colony by breeding in new insects from the wild every few generations; *Colias eurytheme* show severe inbreeding depression, so highly inbred lines are impossible.

In this work, we used *Colias eurytheme* of one standard, functionally intermediate genotype, 3/3, of the enzyme phosphoglucose isomerase (PGI). Natural genetic polymorphs of this enzyme differ by up to fourfold in kinetics and thermal stability (Watt, 1983); these functional differences generate predictable differences in flight fuel supply (Watt and Boggs, 1987) and flight performance (Watt et al., 1983), leading in turn to genotypic fitness-component differences in the wild (e.g. Watt, 1992). By holding PGI genotype constant, we could assess 'baseline' performance of adenylate variation under flight demand and varying T_b; specific genotypic comparisons will follow later.

Flight bioassay

We tested flight in a net enclosure measuring 0.5 m×1 m×1 m in a well-lit temperature-controlled chamber. Butterflies were equilibrated to ambient air temperature, T_a, in jars. Flight was begun by shaking a butterfly out of its jar; it then flew 5–10 cm below the lights. Muscular heat plus energy absorbed from the lights raised T_b to 5 °C above T_a in these conditions, as measured by thermocouple implantation following Tsuji et al. (1986). If flight stopped, it was restarted at once by gentle prodding; flight effort was indexed by the number of stops/restarts. At the end of a predetermined flight duration, the insect was freeze-clamped with liquid nitrogen within 5 s.

Tissue extraction method

To extract adenylates, we at first used 1.325 mol l^{-1} perchloric acid, the classic metabolic extraction reagent (e.g. Sacktor and Wormser-Shavit, 1966). In concentrating adenylate extracts using a centrifugal dryer, residual perchlorate (approximately 0.05 mol l^{-1} in neutralized, precipitated extracts) oxidized other metabolites of interest. Therefore, we substituted 4.2 mol l^{-1} formic acid, giving the same $[H^+]$ as perchloric acid. Formate is not a strong oxidizer and is volatile, hence easily removed during concentration.

Each *Colias eurytheme*, stored in liquid nitrogen (N_2) after freezing, was removed from this to a metal block at 0 °C, on which the thorax (20–40 mg) was isolated and chopped into

pieces using a cold scalpel. Pieces were immediately placed into a 1.5 ml microcentrifuge tube in a metal block at −80 °C. Extraction acid (100 µl) (see above) was added, and the tissue was frozen for 2 min. The tube was moved to an ice-bath for 2 min to nearly thaw the tissue, then returned to the −80 °C block to refreeze. More acid (400 µl) was added at 0 °C, and a Teflon pestle was used to grind the tissue just fast enough that friction maintained a semi-frozen slurry. The slurry was then frozen in liquid N_2, rethawed, refrozen and thawed again to complete extraction. The extract was centrifuged for 15 min at 4–6 °C and 13 000 ***g***, the supernatant volume was noted for dilution corrections, and 400 µl was neutralized with an equal volume of equimolar ammonium hydroxide. To each 800 µl of neutralized extract, 440 µl of 0.5 mol l^{-1} ammonium bicarbonate at pH 8.1 was added to bring the extract pH to a buffered value of 6.5–7.0. The insect's abdomens were kept at −80 °C for electrophoretic genotyping (Carter and Watt, 1988).

Separation and quantitative analysis of flight muscle adenylates by HPLC

Adenylates were separated (Fig. 1) on a 4.6 mm×5 cm Vydac NT 303 ion-exchange column, using a gradient (made using an ISCO pump and gradient former) from solution A (0.016 mol l^{-1} ammonium formate titrated to pH 4.5 with phosphoric acid) to solution B (0.5 mol l^{-1} sodium phosphate titrated to pH 2.7 with formic acid) as follows: 100 % A, 4 min; a linear ramp of 100 % A to 100 % B, 30 min; a ramp back to 100 % A, 1 min; 100 % A, 20 min (flow rate 1 ml min^{-1}). Only ATP, ADP, AMP and NAD^+ were found in thoracic extracts of *Colias eurytheme* (Fig. 1), although other nucleotides ($NADP^+$, GMP, GTP) have been found using this method in sea anemones *Anthopleura elegantissima* (Smith and Watt, 1995). Vydac has now ceased to produce the NT303 column, but similar separation can be performed on a Vydac 302IC column with a gradient from 0.05 to 0.5 mol l^{-1} sodium phosphate at pH 2.8.

Separation was automated using an Alcott 728 autosampler, controlled by ISCO ChemResearch software run on an IBM PS/2-25 microcomputer. Samples were injected into the column after 1 min of the initial 4 min of the gradient. Adenylates were detected at 260 nm (Fig. 1) using an ISCO V^4 ultraviolet monitor, whose signal was processed using an ISCO analog/digital converter and ChemResearch software running on the PS/2. Fig. 2 plots integrated absorbance *versus* adenylate injected, giving extinction coefficients for each adenylate. Extinction coefficients, volume corrections and thoracic weights were applied to raw absorbance data using a processing program written in KnowledgeMan 2.6 (MDBS, Inc.) to give amounts per milligram of thorax, per whole thorax and various ratios.

Assay of inorganic phosphate

Determination of inorganic phosphate, P_i, followed Black and Jones' (1983) modification of the method of Fiske and Subbarow (1925). This method has long been notorious for its high and variable blank readings and annoyingly variable assays. Therefore, following Black and Jones' (1983) successful use of plastic reaction tubes, we compared the use of glass with plastic equipment, including spectrophotometer cuvettes, in all stages of the assay. The best results were obtained when plastic equipment was used throughout the procedure. Residual P_i adsorption to silicate-based materials, not seen with plastic, is the probable cause of the previous unreliability of this assay.

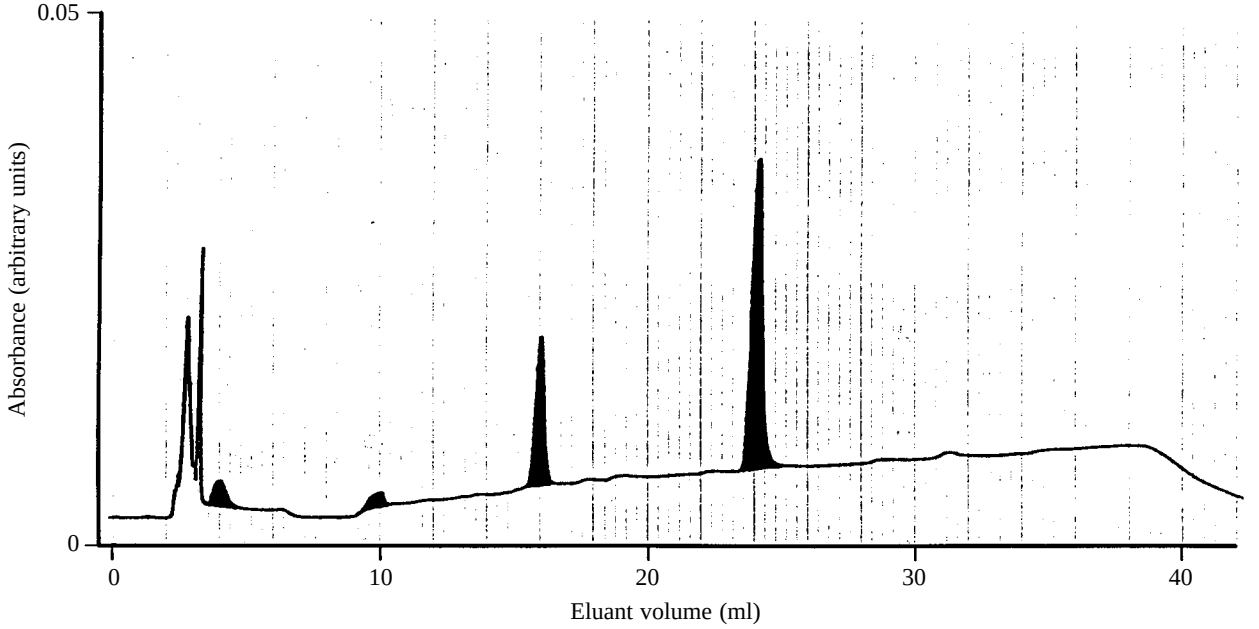

Fig. 1. High-performance liquid chromatography separation of adenylates from a single *Colias eurytheme* thorax. Adenylates were detected at 260 nm; all chemical conditions and instrumentation are as described in the text. Shaded peaks and positions in the separation, left to right: NAD^+ at 4.0 ml, AMP at 9.8 ml, ADP at 16.0 ml and ATP at 24.2 ml.

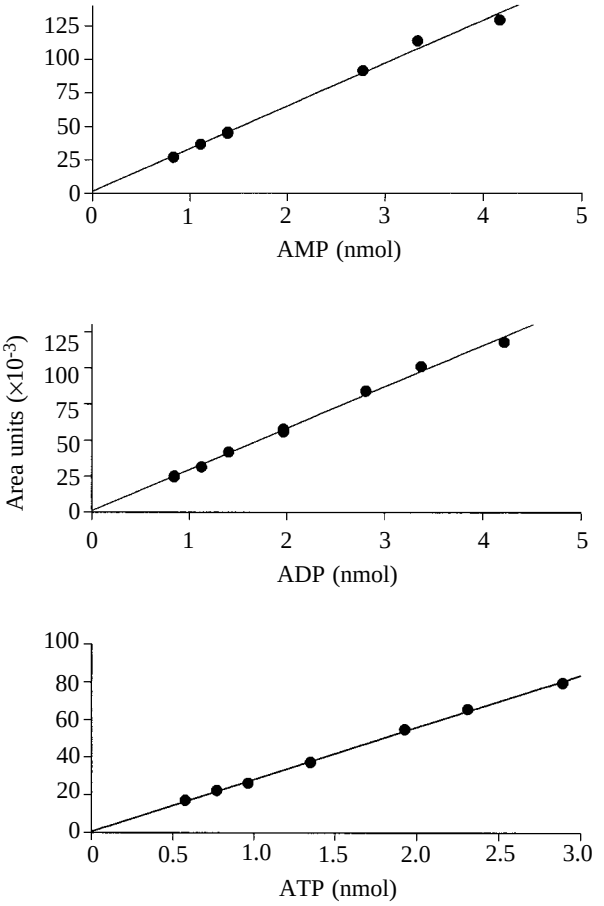

Fig. 2. Calibration plots (integrated absorbance *versus* concentration of adenylate injected) for determining extinction coefficients for quantitative assay of adenylates at 260 nm as described in the text. All linear regressions are statistically significant $P \ll 0.001$. Extinction coefficients for converting absorbance units to nanomoles of adenylate injected are system-specific; they are as follows: AMP 31 893, ADP 28 615, ATP, 27 706.

Reagents were as follows: (reagent A) 0.02 mol l^{-1} (2 % w/v) ammonium molybdate tetrahydrate; (reagent B) 0.80 mol l^{-1} (14 % w/v) ascorbic acid in 3.1 mol l^{-1} (50 % w/v) trichloroacetic acid, TCA; (reagent C) 0.07 mol l^{-1} (2 % w/v) trisodium citrate dihydrate plus 0.15 mol l^{-1} (2 % w/v) sodium arsenite in 0.33 mol l^{-1} (2 % v/v) acetic acid. Reagent B was kept at 0–4 °C and was reprepared at intervals of less than 4 days; other solutions were stable for months at room temperature. Extracts of *Colias eurytheme* to be analyzed for phosphate levels were filtered through Nalgene syringe filters, 0.45 µm pores × 25 mm diameter, and 0.5 ml of 0.18 mol l^{-1} TCA was added to 0.5 ml of each filtered extract. Each 1 ml sample was mixed rapidly in a polystyrene tube with 0.2 ml of reagent A and 0.3 ml of reagent B; after 1 min, 1 ml of reagent C was added and rapidly mixed. Color developed completely in 5 min. Blanks were made with 0.18 mol l^{-1} TCA as 'sample'. Absorbance was read at 700 nm in a Gilford spectrophotometer. A P_i standard was prepared with 0.001 mol l^{-1} sodium phosphate in 0.18 mol l^{-1} (3 % w/v) TCA;

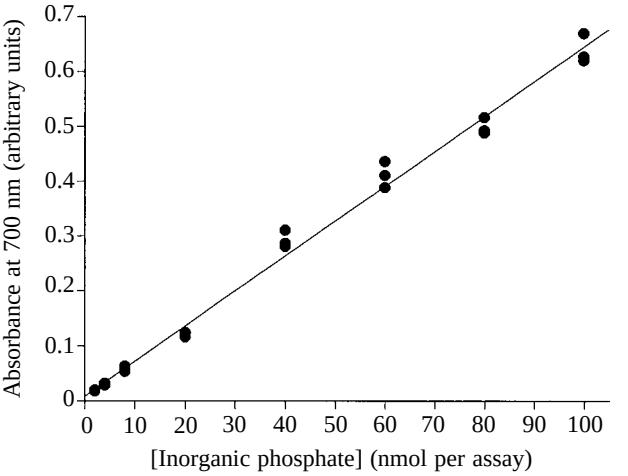

Fig. 3. Calibration plot for the spectrophotometric inorganic phosphate (P_i) assay as described in the text. Absorbance=0.0091+0.006352[P_i]. Statistical test of slope: $F_{1,22}$=2731.6, $P<10^{-6}$.

this was used for calibration over the range 0–100 nmol P_i, as plotted in Fig. 3.

Measures of adenylate pool status and their implications for flight limitation

Several indices of *in vivo* adenylate levels, other than raw concentrations, are available: Atkinson's (1977) 'energy charge', {[ATP]+([ADP]/2)}/([ATP]+[ADP]+[AMP]); the mass action ratio for actomyosin ATPase, [ATP]/([ADP]+[P_i]); or the [ATP]/[ADP] ratio, which often covaries with the ATPase mass action ratio. 'Energy charge', often featured in textbooks, does not control any specific metabolic process; here, we focus on the mass action ratio and [ATP]/[ADP].

When interpreting variation in adenylate levels, one must remember that muscle adenylates are compartmentalized into separate, though connected, pools in the myofibrils *versus* in the wider cytosol (Veech et al., 1979; Balaban, 1984). Organ-level assays, whether based on chemistry or on magnetic resonance, cannot resolve these compartments, but instead yield bulk cytosol values. These values can and do, nonetheless, show the balance between the use and the replenishment of ATP, as studied here.

Statistical analyses

We used SYSTAT 5.0 (Wilkinson, 1990) or FORTRAN programs written to execute tests not covered by this software package (Sokal and Rohlf, 1995; Goldstein, 1964).

Results

Validation and calibration of extraction and assay methods

To compare the effectiveness of 1.35 mol l^{-1} perchloric acid and 4.2 mol l^{-1} formic acid at extracting adenylates, we chose from a single brood of *C. eurytheme* two size-matched groups

Table 1. *Concentrations and correlation patterns among adenylate and inorganic phosphate levels and alternative indices of intramuscular energy status in* Colias eurytheme *flight muscle*

	$[P_i]$ (nmol mg^{-1})	[AMP] (nmol mg^{-1})	[ADP] (nmol mg^{-1})	[ATP] (nmol mg^{-1})	[ATP]/[ADP]	MASACT
Summary data	8.57±0.21	0.30±0.03	1.40±0.05	3.62±0.10	2.86±0.12	0.362±0.020
Correlations						
$[P_i]$		2.4×10^{-5}	$<10^{-6}$	0.67	7.8×10^{-5}	$<10^{-6}$
[AMP]	0.42		$<10^{-6}$	6.3×10^{-3}	$<10^{-6}$	$<10^{-6}$
[ADP]	0.50	0.76		0.22	$<10^{-6}$	4.2×10^{-4}
[ATP]	0.04	–0.28	0.13		$<10^{-6}$	$<10^{-6}$
[ATP]/[ADP]	–0.39	–0.74	–0.71	0.53		$<10^{-6}$
MASACT	–0.56	–0.58	–0.35	0.75	0.79	

Ninety-five individuals were flown for 2 min at T_b=35 °C.

For correlations, the lower left triangular submatrix of values contains Pearson correlation coefficients among variables identified along the margins; the upper right triangular submatrix of values contains *P* values for obtaining each result by chance alone.

MASACT, the whole-cytosol mass action ratio for actomyosin ATPase: [ATP]/([ADP]+$[P_i]$).

See text for correction of test significance levels to adjust for the multiplicity of correlations.

All summary data are means ± S.E.M.

All metabolite concentrations are in nmol mg^{-1} thorax mass.

T_b, body temperature.

of four males each, freeze-clamped them when at rest, extracted the thoraces of one group with 1.35 mol l^{-1} perchloric acid and of the other with 4.2 mol l^{-1} formic acid (see above), then assayed their resting [ATP]/[ADP] ratios. Mean forewing lengths (indexing size) and [ATP]/[ADP] values (±S.E.M.) were: perchloric acid, 2.08±0.07 cm and 3.83±0.05; formic acid, 2.09±0.06 cm and 3.83±0.06. The two acid treatments are of equal extraction effectiveness, as expected.

Accuracy and reproducibility of separation and assay of adenylates over a range of biologically relevant concentrations are crucial. At low sample concentrations, such as is usual for AMP (10–100 pmol per sample), reproducibility was within 2–3 %, with, for example, mean AMP=35.9±0.9 pmol per sample (mean ± S.E.M., *N*=4); at nanomolar levels, reproducibility was approximately 0.1 %, for example ADP=0.491±0.0005 nmol per sample (*N*=4) and ATP=1.89±0.0014 nmol per sample (means ± S.E.M., *N*=4). As a further check, we added 20 nmol samples of pure AMP, ADP and ATP to extracts and examined the additivity of recovery of these samples for eight different extracts compared with their original values. The amounts recovered were: AMP, 19.85±0.22 nmol; ADP, 19.98±0.08 nmol; ATP, 20.06±0.03 nmol (means ± S.E.M.).

Correlation of metabolic pools and metabolite indices

Table 1 presents inorganic phosphate and adenylate concentrations, the [ATP]/[ADP] ratio and actomyosin mass action ratio [ATP]/([ADP]+$[P_i]$), as alternative indices of metabolic status, and their correlations in *Colias eurytheme* flown at T_b=35 °C for 2 min (optimal T_b for flight; within the flight duration for which adenylate concentrations plateau; see below). As *k*=6 correlations among the metabolites are possible, we corrected the usual significance level α=0.05 to a multiple-test level α' using the Dunn–Sidàk method (Sokal and Rohlf, 1995): $\alpha'=1-(1-\alpha)^{1/k}=0.0085$. By this criterion, [ATP] and [AMP] are significantly negatively correlated, and $[P_i]$, [AMP] and [ADP] are positively correlated. The multiplicity of sources for ATP regeneration (arginine phosphate, adenylate kinase and carbohydrate catabolism), with diverse effects on [ADP] and $[P_i]$, accounts for the lack of clear negative correlation between [ATP] and [ADP] or $[P_i]$.

For the nine correlations among two metabolite ratios *versus* the primary metabolites and one another, Dunn–Sidàk α'=0.0057. All correlations were nonetheless significant and those of each ratio conformed to predictions. [ATP]/[ADP] and the actomyosin mass action ratio were very strongly correlated (r=0.79, $P<10^{-6}$; Table 1), making the former ratio attractive as an index of flight-muscle adenylate status compared with the latter, which requires the additional measurement of $[P_i]$.

Dynamics of thoracic adenylate pools in free, interrupted flight

Fig. 4 presents adenylate concentrations and the [ATP]/[ADP] ratio for *Colias eurytheme* from 0 to 60 s of free flight at $T_b\approx35$ °C. Table 2 presents general linear model (GLM) statistics for these data, with brood (pooled in Fig. 4) treated as a category variable and flight time and number of restarts per minute as numeric variables. The number of restarts per minute had no effect on the adenylate levels. Total adenylate levels did not vary with brood or flight time; this ruled out the possible action of the ‘purine nucleotide cycle’ (e.g. Mommsen and Hochachka, 1988). [ATP] and [ATP]/[ADP] declined significantly in the first minute of flight, while [AMP] increased significantly (Fig. 4). Table 3 shows that extending the flight time to 1–6 min did not produce further changes in the adenylate pools.

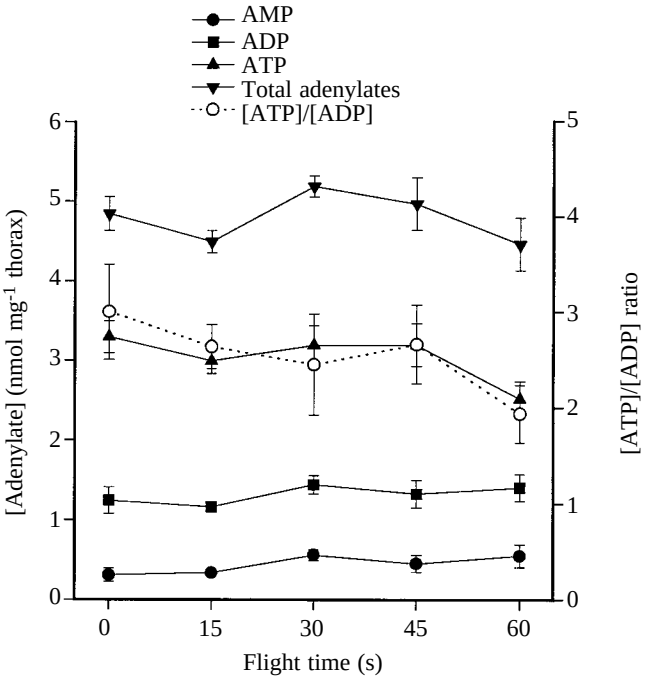

Fig. 4. Thoracic flight-muscle adenylate levels and [ATP]/[ADP] during the first minute of flight by *Colias eurytheme* at a body temperature of 35 °C. *N*=7 insects for each time point except 60 s, where *N*=6. Two broods were pooled. Statistical analysis is given in Table 2.

Table 2. *General linear model analyses of adenylate variation in the first minute of* Colias eurytheme *flight*

Variable	Source	d.f.	Mean square	*F*-ratio	*P*
[ANP]	Brood	1	0.320	0.717	0.40
	Flight time	1	0.002	0.006	0.94
	Restarts per minute	1	0.046	0.103	0.75
	Error	30	0.446		
[AMP]	Brood	1	0.188	3.742	0.06
	Flight time	1	0.289	5.761	0.02
	Restarts per minute	1	0.081	1.617	0.21
	Error	30	0.050		
[ADP]	Brood	1	0.293	2.432	0.13
	Flight time	1	0.314	2.606	0.12
	Restarts per minute	1	0.307	2.547	0.12
	Error	30	0.120		
[ATP]	Brood	1	2.350	8.246	0.007
	Flight time	1	1.292	4.531	0.04
	Restarts per minute	1	0.380	1.335	0.26
	Error	30	0.285		
[ATP]/[ADP] (transformed)	Brood	1	1.44×10^{-4}	5.864	0.02
	Flight time	1	1.22×10^{-4}	4.975	0.03
	Restarts per minute	1	0.61×10^{-4}	2.498	0.12
	Error	30	0.25×10^{-4}		

Data from Fig. 4, taken at T_b=35 °C, are analyzed. [ANP] is the sum of all three nucleotide pools: [AMP]+[ADP]+[ATP].

[ATP]/[ADP] was subjected to arcsin-square-root transformation as appropriate for least-squares analysis of ratios.

Flight effort level as indexed by number of restarts per minute

We analyzed the relationship between adenylate pools and insect 'effort level' as measured by the nmber of restarts per minute in our flight assay. Restarts varied with brood and with T_b: e.g. in the animals whose adenylate variation is analyzed in Table 3, the average number of restarts per minute ranged from 4.04 in brood 2_6O to 14.1 in brood 21_{14}A. At T_b=31 °C, the number of restarts was 10.6±0.9; at T_b=35 °C, 10.1±0.7; at T_b=39 °C, 11.2±0.6 (means ± S.E.M., *N* as in Table 3). GLM analysis of these restart data showed that the effect of flight time was not significant, that the effect of brood difference was significant ($F_{6,103}$=3.69, *P*=0.0023) and that the effect of T_b differences nested under broods was weakly significant ($F_{8,103}$=2.15, *P*=0.037). *Colias eurytheme* demonstrating sluggish flight, i.e. with high restart values, have higher [ATP] and [ATP]/[ADP], probably because of reduced cytosol ATP drawdown by highly interrupted flight, but there is no clearer covariation between the number of restarts and [ATP] or its ratios (Tables 2, 3). In any event, sluggish *Colias eurytheme* are poor subjects for studying the effects of flight T_b on adenylate levels.

Adenylate levels at diverse body temperatures: testing theories of animal thermoregulation

As noted above, the 'classical' or 'maxithermal' explanation for animal thermoregulation predicts that metabolic throughput should increase with T_b up to the lower limits of thermal damage. If so, then, as *Colias eurytheme* T_b rises from the suboptimum, to the base of the thermal optimum for flight, to the top of the thermal optimum for flight, [ATP] and [ATP]/[ADP] should not only rise into the thermal optimum, but continue to rise or at least to maintain a maximum value across the thermal optimum right up to denaturation temperatures. (The behavioral optimum would then result from adjustment of behavior to maintain T_b below damage temperatures or from the thermal dependence of biophysical limitations, such as muscle elasticity or cuticular structure.)

The hypothesis of constraint on the thermal breadth of metabolic performance proposes that the maximal speed of response to biological demand in metabolic pathways cannot evolve to span more than a few degrees, because the multiplicity of reaction steps, each with its own thermal dependence, must be co-adjusted by evolution to achieve maximal performance (Watt, 1991). If so, then as T_b rises, [ATP] and [ATP]/[ADP] values should pass through peak values within the thermal optimum, then decrease again well before the lower limits of thermal damage are reached.

To test these hypotheses, we flew *Colias eurytheme* at

Table 3. *Test of alternative hypotheses relating metabolic performance to* T_b *and* Colias eurytheme *thermoregulation*

Data summary

	[ATP]/[ADP]		
Brood	31 °C	35 °C	39 °C
2_{28}O	1.11±0.13 (5)		2.29±0.50 (4)
2_{6}O	0.75±0.03 (2)		1.65±0.19 (5)
2_{8}O		4.14±0.43 (7)	1.80±0.20 (7)
21_{14}A		3.27±0.26 (7)	2.41±0.28 (6)
28_{14}A		2.87±0.37 (8)	2.45±0.37 (6)
3_{0}O	2.56±0.23 (15)	3.11±0.34 (14)	2.01±0.19 (18)
7_{11}O		2.30±0.19 (9)	1.81±0.19 (8)
All	2.30±0.23 (20)	3.04±0.19 (45)	2.03±0.10 (54)

Nested general linear model

Source	d.f.	Mean square	*F*-ratio	*P*
Flight time (s)	1	3.10×10^{-5}	1.31	0.25
Restarts per minute of flight	1	0.6×10^{-7}	0.03	0.87
Brood	6	1.05×10^{-4}	4.44	0.0005
T_b nested under brood	8	1.19×10^{-4}	5.06	2.59×10^{-5}
Error	102	2.36×10^{-5}		

[ATP]/[ADP] was subjected to arcsin-square-root transformation before general linear model analysis, as appropriate for a ratio (data are summarized untransformed).

Insects were flown at two or three body temperatures (T_b) per brood as indicated.

Predictions of hypotheses tested are given in the text.

Flight times vary in some broods between 60 and 360 s, but without significant effect.

The number of restarts per minute (data not given) was tested and was without effect.

Brood effects are highly significant, as in other experiments.

[ATP]/[ADP] increases from T_b=31 °C to T_b=35 °C, then falls again at T_b=39 °C.

See text for further discussion.

Values are means ± S.E.M. (*N*).

T_b=31 °C in the thermal suboptimum for flight, at 35 °C at the base of the optimum, and at 39 °C at the top of the optimum (see Watt, 1968). The 'maxithermal' hypothesis predicted that [ATP] and [ATP]/[ADP] should be the same or higher at 39 °C as at 35 °C, while the metabolic-constraint hypothesis predicted that [ATP] and [ATP]/[ADP] should be the same or lower at 39 °C as at 35 °C. Both hypotheses predict that [ATP] and [ATP]/[ADP] should be higher at T_b=35 °C than at 31 °C.

T_b=39 °C, at the top of the thermal optimum, is below damaging temperatures (Watt, 1968; Kingsolver and Watt, 1983). Nonetheless, we ran an experiment to control directly against damage effects at 39 °C. We first flight-tested *Colias eurytheme* from several broods at 39 °C, allowed them to rest for 10 min or 1 h, and then reflew them at 35 °C and freeze-clamped them as usual. Siblings from each brood were simply flown at 35 °C and freeze-clamped as controls. As Table 4 shows, there was no significant difference in [ATP]/[ADP] in flight at 35 °C between the experimentals and the controls. This excludes any possible confounding of our design by thermal damage.

Table 3 analyzes data from seven broods flown at the three T_b values and for flight times ranging from 1 to 6 min. Flight time and the number of restarts per minute did not significantly affect variation in flight-muscle [ATP]/[ADP]; broods varied greatly in maintenance levels of the adenylates. T_b nested under brood had highly significant effects, and the results clearly discriminate between the two primary explanations for thermoregulation: [ATP]/[ADP] was already sharply reduced by the top of the behavioral thermal optimum for flight, at 39 °C, compared with 35 °C. This rejects the 'maxithermal' metabolic explanation for thermoregulation and is consistent with the 'narrow-thermal-bandwidth' constraint hypothesis.

Discussion

Comparative physiology and further study of adenylate dynamics

The thoracic adenylate concentrations and dynamics of *Colias eurytheme* are similar to those of *Phormia regina* (Sacktor and Hurlbut, 1966), although displaying slightly higher concentrations of P_i (8.6 *versus* 7.6 nmol mg^{-1} thorax) and AMP (0.3 *versus* 0.26 nmol mg^{-1} thorax), and approximately 30 % lower [ADP] and [ATP] (1.4 *versus* 1.95 nmol mg^{-1} thorax and 3.6 *versus* 6.3 nmol mg^{-1} thorax, respectively). In *Phormia regina*, [P_i] and [AMP] showed clear overshoots and settled to steady-state in the first minute of flight, and levels of some adenylates showed hints of lower-amplitude oscillation for some time after the onset of flight (Sacktor and Hurlbut, 1966); levels of carbohydrate metabolites clearly oscillated (Sacktor and Wormser-Shavitt, 1966). Our data do not show such dynamics when pooled by brood (Fig. 4), but in separate broods, hints of oscillation do appear. Such dynamics may be clearer at sub- or hyper-optimal T_b, but should also be more evident in continuous flight. Sacktor and Hurlbut (1966) and Sacktor and Wormser-Shavit (1966) flew *Phormia regina* continuously for chosen durations on a circular 'flight mill' under stable, although unspecified, thermal conditions. We are developing a continuous-flight assay, using a computerized flight balance in which T_b can be controlled and monitored and lift and thrust can be recorded (M. R. Dohm, W. B. Watt and R. Conn, unpublished results; see also Esch, 1976). This technology will allow more detailed analyses of the interaction of metabolic dynamics with flight.

Further aspects of metabolic adaptation or constraint in thermoregulatory evolution

The decrease in [ATP]/[ADP] between T_b=35 °C and T_b=39 °C, which rules out the 'maxithermal' explanation of thermoregulation, may be due to limitation by one or a few steps in the ATP resupply metabolism or to a constraint spread more evenly among all steps. We can test these alternatives by studying the thermal dependence of the diverse mechanisms for ATP resupply. The adenylate kinase reaction and the

Table 4. *Test for thermal damage to metabolism as a possible result of* Colias eurytheme *flight at 39 °C*

Data summary

Brood	Flight/reflight temperature (°C)	Reflight interval (min)	*N*	[ATP]/[ADP] Group	[ATP]/[ADP] Brood	[ATP]/[ADP] Temperature (°C)
6_0O	39/35	10	7	1.97±0.30		
					1.69±0.17	
	35		8	1.63±0.20		
						39/35:
7_0O	39/35	10	5	2.91±0.48		2.78±0.21
					2.95±0.37	
	35		5	3.20±0.61		
						35:
14_1A	39/35	60	9	3.07±0.27		2.59±0.29
					3.42±0.26	
	35		9	3.48±0.45		

Analysis of variance

Source	d.f.	Mean square	*F*-ratio	*P*
Brood	2	2.67×10^{-4}	10.29	0.0003
Rest interval	1	1.44×10^{-5}	0.55	0.46
Flight/reflight temperature	1	0.6×10^{-5}	0.23	0.63
Error	38	2.60×10^{-5}		

Experimental insects were flown for 2 min at T_b=39 °C, then rested for either 10 min or 60 min, reflown for 2 min at T_b=35 °C, then freeze-clamped. Controls were flown for 2 min at T_b=35 °C and freeze-clamped.

[ATP]/[ADP] was subjected to arcsin-square-root transformation before general linear model analysis, as appropriate for a ratio (this explains the seemingly small mean-square values; data are summarized untransformed).

Broods differ in [ATP]/[ADP] as in other experiments; neither the duration of rest before reflight, nor the flight regime itself, has a significant effect.

No damage is found; see text for further details.

Values are means ± S.E.M.

recharging of ATP from arginine phosphate are each catalyzed by single enzymes. If either has low, sharp upper thermal limits on function, enzymology should reveal it. If ATP resupply by carbohydrate metabolism contains one or a few sharply limiting steps, the reduction in performance between 35 and 39 °C should be localizable to these by studies of metabolic fluxes or pools at intervals in the system. In contrast, a 'uniform constraint' metabolic model predicts that each reaction step alone may have a broader thermal performance, but that the performances of pathway subsystems will be narrowed by multiplicatively poorer performance of the sequence of steps outside a common thermal maximum (e.g. glycolytic performance will decrease between 35 and 39 °C in the same way as the whole ATP resupply system).

The 33 % decrease in [ATP]/[ADP] between 35 and 39 °C (Table 3) also suggests why 39 °C is the top of the flight maximum. If the decrease continues above 39 °C, flying insects may risk exhausting their ATP resupply capacity, becoming trapped in overheating conditions and susceptible to damage at even higher T_b. (All flight observed above T_b=39 °C is directed towards shade and, hence, escape from solar overheating; Watt, 1968.) The mechanisms of thermal damage, when it occurs, and acute response to it thus require study. In *C. eurytheme*, exposure to 45 °C sterilizes females and drastically shortens male lifespan (Kingsolver and Watt, 1983). Momentary T_b values as high as 47 °C have been recorded in live *C. alexandra* temporarily trapped in intense sunlight at high T_a (Watt, 1968). The effects of such brief overheating are not yet known, but they may invoke the function of protein-structure chaperones such as heat-shock proteins (e.g. hsp70) (see Krebs and Feder, 1997).

All these questions should be experimentally testable in the future.

An implication for evolutionary genetic studies

Genetic variants of the enzyme phosphoglucose isomerase (PGI) in *Colias* spp. differ in kinetics and thermal stability by up to three- to fourfold (Watt, 1983). These large differences, in the context of metabolic network theory, predict genotype-specific impacts on the glycolytic metabolism of their carriers as transmitted through the flight-muscle adenylate pools and, hence, on flight performance (Watt, 1983; Watt et al., 1983). In turn, because all adult fitness components depend on flight, these flight differences predict large genotype-specific differences in survivorship, male mating success and female fecundity. All these

predictions have been repeatedly tested and sustained in field experiments (Watt, 1992; Watt et al., 1983, 1985, 1986). These effects are, as expected, manifested against large background variances, unrelated to the PGI variants (e.g. Watt, 1992). The large brood-specific differences in adenylate resupply seen in our experiments imply a large broad-sense background heritability of difference in metabolic performance, which may be a large part of this natural background variance in flight performance and consequent fitness components. This offers a fine chance to explore interactions among major genes (i.e. genes for PGI) and genetic background, as each affects adenylate resupply.

Implications for evolutionary responses to environmental warming

Our 'narrow thermal bandwidth' explanation for animal thermoregulation based on metabolic organization implies that natural selection may not be able to adjust the thermal specialization of high-demand metabolic processes quickly. This is because selection would have to alter the thermal kinetics of many different gene products simultaneously, assuming relevant genetic variation existed at all such genes in affected populations. Here, we would have the 'catastrophic change' version of Haldane's (1957) 'cost of evolution' paradox: if anthropogenic causes (e.g. Kareiva et al., 1993) change thermal environments more quickly than animals can evolve in response, massive population decreases or even extinctions may occur. Thus, this line of study may illuminate practical problems of environmental management under potential global change as well as basic issues in the functional ecology and evolution of physiological mechanisms.

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References

Atkinson, D. E. (1977). *Cellular Energy Metabolism and its Regulation*. New York: Academic Press.

Bailey, E. (1975). Biochemistry of insect flight: fuel supply. In *Insect Biochemistry and Function* (ed. D. J. Candy and B. A. Kilby), pp. 89–176. London: Chapman & Hall.

Balaban, R. S. (1984). The application of nuclear magnetic resonance to the study of cellular physiology. *Am. J. Physiol.* **246**, 10–19.

Bartholomew, G. (1981). A matter of size: an examination of endothermy in insects and terrestial vertebrates. In *Insect Thermoregulation* (ed. B. Heinrich), pp. 45–78. New York: J. Wiley & Sons.

Bartholomew, G. (1987). Interspecific comparison as a tool for ecological physiologists. In *New Directions in Ecological Physiology* (ed. M. E. Feder, A .F. Bennett, W. W. Burggren and R. B. Huey), pp. 11–35. Cambridge: Cambridge University Press.

Black, M. J. and Jones, M. E. (1983). Inorganic phosphate determination in the presence of a labile organic phosphate: assay for carbamyl phosphate phosphatase activity. *Analyt. Biochem.* **135**, 233–238.

Carter, P. A. and Watt, W. B. (1988). Adaptation at specific loci. V. Metabolically adjacent enzyme loci may have very distinct experiences of selective pressures. *Genetics* **119**, 913–924.

Casey, T. M. (1981). Insect flight energetics. In *Locomotion and Energetics in Arthropods* (ed. C. F. Herreid and C. R. Fourtner), pp. 419–452. New York: Plenum Press.

Crawford, D. L. and Powers, D. A. (1989). Molecular basis of evolutionary adaptation at the lactate dehydrogenase-B locus in the fish *Fundulus heteroclitus*. *Proc. Natl. Acad. Sci. USA* **86**, 9365–9369.

Ellington, C. P., Machin, K. E. and Casey, T. M. (1990). Oxygen consumption of bumblebees in forward flight. *Nature* **347**, 472–473.

Esch, H. (1976). Body temperature and flight performance of honeybees in a servomechanically controlled wind tunnel. *J. Comp. Physiol.* A **109**, 265–277.

Fiske, C. H. and Subbarow, Y. (1925). The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**, 375–400.

Gmeinbauer, R. and Crailsheim, K. (1993). Glucose utilization during flight of honeybee (*Apis mellifera*) workers, drones and queens. *J. Insect Physiol.* **39**, 959–967.

Goldstein, A. (1964). *Biostatistics*. New York: Macmillan.

Haldane, J. B. S. (1957). The cost of natural selection. *J. Genet.* **55**, 511–524.

Heinrich, B. (1974). Thermoregulation in endothermic insects. *Science* **185**, 747–756.

Heinrich, B. (1977). Why have some animals evolved to regulate a high body temperature? *Am. Nat.* **111**, 623–640.

Heinrich, B. (1981). Ecological and evolutionary perspectives. In *Insect Thermoregulation* (ed. B. Heinrich), pp. 235–302. New York: J. Wiley & Sons.

Josephson, R. K. (1981). Temperature and mechanical performance of insect muscle. In *Insect Thermoregulation* (ed. B. Heinrich), pp. 19–44. New York: J. Wiley & Sons.

Kareiva, P., Kingsolver, J. G. and Huey, R. B. (1993). *Biotic Interactions and Global Change*. Sunderland, MA: Sinauer Ass.

Kingsolver, J. G. (1983a). Thermoregulation and flight in *Colias* butterflies: elevational patterns and mechanistic limitations. *Ecology* **64**, 534–545.

Kingsolver, J. G. (1983b). Ecological significance of flight activity in *Colias* butterflies: implications for reproductive strategy and population structure. *Ecology* **64**, 545–551.

Kingsolver, J. G. and Watt, W. B. (1983). Thermoregulatory strategies in *Colias* butterflies: thermal stress and the limits to adaptation in thermally varying environments. *Am. Nat.* **121**, 32–55.

Krebs, R. A. and Feder, M. E. (1997). Natural variation in the expression of the heat-shock protein *hsp70* in a population of *Drosophila melanogaster* and its correlation with tolerance of ecologically relevant thermal stress. *Evolution* **50**, 173–179.

Mommsen, T. P. and Hochachka, P. W. (1988). The purine nucleotide cycle as two temporally separated metabolic units: a study on trout muscle. *Metabolism* **37**, 552–556.

Sacktor, B. (1975). Biochemistry of insect flight: utilization of fuel by muscles. In *Insect Biochemistry and Function* (ed. D. J. Candy and B. A. Kilby), pp. 1–88. London: Chapman & Hall.

Sacktor, B. and Hurlbut, E. C. (1966). Regulation of metabolism in working muscle *in vivo*. II. Concentrations of adenine nucleotides,

arginine phosphate and inorganic phosphate in insect flight muscle during flight. *J. Biol. Chem.* **241**, 632–634.

Sacktor, B. and Wormser-Shavit, E. (1966). Regulation of metabolism in working muscle *in vivo*. I. Concentrations of some glycolytic, tricarboxylic acid cycle and amino acid intermediates in insect flight muscle during flight. *J. Biol. Chem.* **241**, 624–631.

Smith, B. L. and Watt, W. B. (1995). Adenylate levels and environmental stress in the sea anemone *Anthopleura elegantissima*. *Mol. Mar. Biol. Biotechnol.* **3**, 261–269.

Sokal, R. R. and Rohlf, F. J. (1995). *Biometry*. San Francisco: W. H. Freeman & Company.

Stucki, J. W. (1980a). The thermodynamic buffer enzymes. *Eur. J. Biochem*. **109**, 257–267.

Stucki, J. W. (1980b). The optimal efficiency and the economic degrees of coupling of oxidative phosphorylation. *Eur. J. Biochem*. **109**, 269–283.

Tsuji, J. S., Kingsolver, J. G. and Watt, W. B. (1986). Thermal physiological ecology of *Colias* butterflies in flight. *Oecologia* **69**, 161–170.

Veech, R. C., Lawson, J. W. R., Cornell, N. W. and Krebs, H. A. (1979). Cytosolic phosphorylation potential. *J. Biol. Chem.* **254**, 6538–6547.

Watt, W. B. (1968). Adaptive significance of pigment polymorphisms in *Colias* butterflies. I. Variation of melanin pigment in relation to thermoregulation. *Evolution* **22**, 437–458.

Watt, W. B. (1969). Adaptive significance of pigment polymorphisms in *Colias* butterflies. II. Thermoregulation and photoperiodically controled melanin variation in *Colias eurytheme*. *Proc. Natl. Acad. Sci. USA* **63**, 767–774.

Watt, W. B. (1983). Adaptation at specific loci. II. Demographic and biochemical elements in the maintenance of the *Colias* PGI polymorphism. *Genetics* **103**, 691–724.

Watt, W. B. (1986). Power and efficiency as indexes of fitness in metabolic organization. *Am. Nat.* **127**, 629–653.

Watt, W. B. (1991). Biochemistry, physiological ecology and population genetics – the mechanistic tools of evolutionary biology. *Funct. Ecol.* **5**, 145–154.

Watt, W. B. (1992). Eggs, enzymes and evolution: natural genetic variants change insect fecundity. *Proc. Natl. Acad. Sci. USA* **89**, 10608–10612.

Watt, W. B. (1997). Accuracy, anecdotes and artifacts in the study of insect thermal ecology. *Oikos* **80**, 399–400.

Watt, W. B. and Boggs, C. L. (1987). Allelic isozymes as probes of evolution of metabolic organization. Isozymes. *Curr. Topics Biol. Med. Res.* **15**, 27–47.

Watt, W. B., Carter, P. A. and Blower, S. M. (1985). Adaptation at specific loci. IV. Differential mating success among glycolytic allozyme genotypes of *Colias* butterflies. *Genetics* **109**, 157–175.

Watt, W. B., Carter, P. A. and Donohue, K. (1986). Females' choice of "good types" as mates is promoted by an insect mating system. *Science* **233**, 1187–1190.

Watt, W. B., Cassin, R. C. and Swan, M. S. (1983). Adaptation at specific loci. III. Field behavior and survivorship differences among *Colias* PGI genotypes are predictable from *in vitro* biochemistry. *Genetics* **103**, 725–739.

Wegener, G., Bolas, N. M. and Thomas, A. A. (1991). Locust flight metabolism studies *in vivo* by phosphorus-31 NMR spectroscopy. *J. Comp. Physiol.* B **161**, 247–256.

Wilkinson, L. (1990). *Systat (5.0): The System for Statistics*. Evanston, IL: SYSTAT, Inc.