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Allometric scaling of flight energetics in orchid bees: evolution of flux capacities and flux rates

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Summary

The evolution of metabolic pathways involved in energy production was studied in the flight muscles of 28 species of orchid bees. Previous work revealed that wingbeat frequencies and mass-specific metabolic rates decline in parallel by threefold as body mass increases interspecifically over a 20-fold range. We investigated the correlated evolution of metabolic rates during hovering flight and the flux capacities, i.e. V_{max} values, of flight muscle enzymes involved in substrate catabolism, the Krebs cycle and the electron transport chain. V_{max} at the hexokinase (HK) step scales allometrically with an exponent almost identical to those obtained for wingbeat frequency and mass-specific metabolic rate. Analysis of this relationship using phylogenetically independent contrasts supports the hypothesis of correlated evolution between HK activity and mass-specific metabolic rate. Although other enzymes scale allometrically with respect to body mass, e.g. trehalase, glycogen phosphorylase and citrate synthase, no other enzyme activities were correlated with metabolic rate after controlling for phylogenetic relatedness. Pathway flux rates were used with enzyme V_{max} values to estimate fractional velocities (fraction of V_{max} at which enzymes operate) for various reactions to gain insights into enzyme function and how this varies with body mass. Fractional velocity is highly conserved across species at the HK step, but varied at all other steps examined. These results are discussed in the context of the regulation and evolution of pathways of energy metabolism.

Key words: enzyme activity, metabolic rate, evolution, allometry, insect flight, orchid bee.

Introduction

Insects in flight achieve the highest known mass-specific metabolic rates in the animal kingdom (Sacktor, 1976). It has been known for many years that the high power outputs required for flight are made possible by the operation of a contractile machinery supplied with ATP from obligately aerobic bioenergetic pathways, endowed with high capacities for enzymatic flux, i.e. V_{max} values (Crabtree and Newsholme, 1972). More recent studies on honeybees *Apis mellifera* have revealed close matches between enzymatic flux capacities and metabolic flux rates during flight at various steps in muscle energy metabolism (Suarez et al., 1996, 2000; Staples and Suarez, 1997; Suarez, 2000). In light of such results, an important question is how flux capacities have evolved in relation to physiological flux rates during the adaptive radiation of flying insects. In a previous study (Darveau et al., 2005), we investigated the evolutionary relationships between form and function associated with hovering flight in a lineage of orchid bees (Apidae; Euglossini). The correlated evolution found among wing loading, wingbeat frequency and mass-specific metabolic rate revealed the consequences of a 20-fold variation in body mass on flight energetics. The threefold range in mass-specific metabolic rate found within this clade of bees provides a means by which to evaluate the relationships between the evolution of metabolic rate and the design of metabolic pathways. The use of a comparative phylogenetic approach (Garland and Carter, 1994; Feder et al., 2000) provides an opportunity to understand adaptive variation in energy metabolism in an evolutionary framework.

In analyses of the quantitative design of metabolic pathways, enzyme maximal velocities, i.e. V_{max} values ($k_{cat}\times$[E], where k_{cat} is the catalytic efficiency and [E] is enzyme concentration), measured *in vitro*, provide useful estimates of the maximum capacities for flux at various steps (Newsholme and Crabtree, 1986; Suarez et al., 1997). As orthologous enzymes adapted for function at similar temperatures display similar k_{cat} values (Hochachka and Somero, 2002), V_{max} values serve as indirect measures of [E]. In addition, V_{max} values, when considered in relation to pathway flux rates, provide insights into enzyme function *in vivo*. Comparisons between flux capacities and flux rates yield valuable information concerning the degree to

which biochemical capacities are matched to physiological loads (Suarez et al., 1996; Suarez, 2000). Application of such analyses to honeybee flight muscles revealed that their high mass-specific metabolic rates during flight require high V_{max} for glycolysis and the electron transport chain, and that certain enzymes operate close to their maximal capacities *in vivo* (Suarez et al., 1996; Suarez, 2000). At near-equilibrium reactions, V_{max} values are typically much greater than net flux rates. Staples and Suarez (1997) found, at the phosphoglucoisomerase step, that the V_{max} value is close to that required to catalyze the required rate of forward flux while maintaining near-equilibrium.

In this study, we investigated the quantitative design of flight muscle metabolic pathways in orchid bees by measuring the activities of selected enzymes involved in fuel breakdown, glycolysis, redox balance and mitochondrial oxidative metabolism. The relationships between body mass, hovering flight metabolic rate and enzyme activity are analysed using the method of phylogenetically independent contrasts (Felsenstein, 1985; Garland et al., 1992). Incorporating this study with our previous analysis of the evolution of orchid bee flight energetics (Darveau et al., 2005), we analyze the correlated evolution between morphological and biomechanical variables with metabolic and biochemical variables associated with hovering flight.

Materials and methods

Orchid bee collection

Orchid bees (Apidae; Euglossini) were captured on Barro Colorado Island at the Smithsonian Tropical Research Institution in Panama. A complete list of species used can be found in Darvaeu (2004). Only males were used, as these could be attracted to and captured after they land on paper moistened with cineole, skatol or methyl salicylate (Sigma Chemicals, Oakville, ON, Canada). These chemicals are found naturally in the fragrances that male orchid bees collect from various floral and non-floral sources (Roubik and Ackerman, 1987).

Enzyme activities

Bees collected for enzyme measurements were frozen at –80°C, shipped in dry ice to the laboratory, and stored at –80°C until measurements were conducted. Individual thoraxes were minced with scissors and homogenized in 19 volumes of ice-cold buffer. All further manipulations were carried out in glass or plasticware cooled in crushed ice. The homogenization buffer used on samples for the measurement of hexokinase (HK), phosphofructokinase (PFK), glycerol 3-phosphate dehydrogenase (GPDH), citrate synthase (CS) and cytochrome oxidase (COX) consisted of 25 mmol l^{-1} Tris-potassium phosphate pH 7.8 at 4°C, 2 mmol l^{-1} ethylene diamine tetra-acetic acid (EDTA), 5 mmol l^{-1} dithiothreitol (DTT), 1 mmol l^{-1} fructose 6-phosphate, 3.5 mmol l^{-1} glucose 6-phosphate and 0.5% (v/v) Triton X-100. The use of phosphate buffer and inclusion of sugar phosphates served to stabilize PFK activity that would otherwise have been lost (Suarez et al., 1996; Wegener et al., 1986). The homogenization buffer used for samples designated for measurement of glycogen phosphorylase (GP), trehalase (TR), and phosphoglucoisomerase (PGI), consisted of 25 mmol l^{-1} Hepes, pH 7.3 at 4°C, 2 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT and 0.5% (v/v) Triton X-100. Minced thoraxes were homogenized three times for 10 s at 30 s intervals, using a Polytron homogenizer with a small tip (Brinkmann Instruments, Rexdale, ON, Canada). Homogenates were then sonicated using a Kontes Micro Ultrasonic Cell Disrupter (Mandel Scientific, Guelph, ON, Canada), again three times for 10 s, at 30 s intervals. Homogenates were centrifuged (Jouan MR 1812, Winchester, VI, USA) for 5 min at 8000 ***g*** at 4°C, and the supernatants used for assays. To ensure that these procedures resulted in complete extraction of membrane-bound enzymes (e.g. trehalase), preliminary studies were conducted to compare enzyme activities in uncentrifuged homogenates and supernatant fractions. Activities obtained were equal, showing that extraction of all enzymes was complete.

Enzyme activities were measured in duplicate using a Perkin-Elmer Lambda 2 UV-Visible spectrophotometer (Norwalk, CT, USA) equipped with a Lauda circulating water bath (Brinkman Instruments) adjusted to maintain cuvette temperatures (monitored using a temperature probe) at 37°C. HK, PFK, GPDH, PGI, TR, GP reactions were monitored by following the rate of appearance or disappearance of reduced nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm using a millimolar extinction coefficient (ϵ) of 6.22. The CS reaction was monitored 5,5′ dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm using ϵ=13.6. The COX reaction was measured by monitoring oxidized cytochrome *c* at 550 nm using ϵ=29.5. Control (background) rates, obtained without one substrate (indicated below), were measured and subtracted from rates obtained with all substrates present.

Assay conditions and substrate concentrations required to elicit V_{max} were as follows: HK: 50 mmol l^{-1} Hepes, pH 7.0, 5 mmol l^{-1} D-glucose (omitted from control), 4 mmol l^{-1} ATP, 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 0.5 mmol l^{-1} $NADP^+$, 5 mmol l^{-1} DTT, 1 U glucose 6-phosphate dehydrogenase. PFK: 50 mmol l^{-1} Tris-HCl, pH 8.0, 5 mmol l^{-1} fructose 6-phosphate (omitted from control), 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 2 mmol l^{-1} ATP, 0.15 mmol l^{-1} NADH, 0.01 mmol l^{-1} fructose 2,6-bisphosphate, 5 mmol l^{-1} DTT, 1 U aldolase, 5 U triosephosphate isomerase, 5 U glyceraldehyde 3-phosphate dehydrogenase. GPDH: 50 mmol l^{-1} imidazol pH 7.0, 1 mmol l^{-1} dihydroxyacetonephosphate (omitted from control), 0.15 mmol l^{-1} NADH. CS: 50 mmol l^{-1} Tris-HCl, pH 8.0, 0.5 mmol l^{-1} oxaloacetate (omitted from control), 0.3 mmol l^{-1} acetyl-CoA, 0.1 mmol l^{-1} DTNB. COX: 50 mmol l^{-1} potassium phosphate, pH 7.5, 0.05 mmol l^{-1} reduced cytochrome *c*. TR: 50 mmol l^{-1} potassium phosphate pH 6.6, 1.1 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} $NADP^+$, 1.1 mmol l^{-1} ATP, 10 mmol l^{-1} trehalose (omitted from control), 2.5 U of hexokinase and glucose 6-phosphate

dehydrogenase. GP: 100 mmol l^{-1} potassium phosphate pH 7.4, 2 mg ml^{-1} glycogen, 0.5 mmol l^{-1} $NADP^+$, 4 μmol l^{-1} glucose 1,6-biphosphate, 2 mmol l^{-1} AMP, 10 mmol l^{-1} $MgCl_2$, 10 U phosphoglucomutase and 2.5 U glucose 6-phosphate dehydrogenase. PGI: 50 mmol l^{-1} Tris-HCl, pH 8.0, 0.5 mmol l^{-1} fructose 6-phosphate, 0.5 mmol l^{-1} $NADP^+$, 2.5 U glucose 6-phosphate dehydrogenase. All chemicals were from Sigma Chemical Company (Oakville, ON, Canada).

Respiration rate measurements in vitro

As individual bees do not possess sufficient flight muscle mitochondria for isolation, we used crude homogenates of individual thoraxes to measure rates of substrate oxidation *in vitro*. Bees were captured in the field and placed in a refrigerator at 4°C until used for measurements. Before preparing the thoraxes for homogenization, individual bees had to be warmed up until leg movements were noticeable. For reasons that remain unknown, this warm-up step was required prior to dissection and thoracic homogenization for O_2 consumption to occur. Preparation of homogenates from cold thoraxes resulted in no detectable respiration. After each thorax was dissected from the insect, further manipulations were performed on ice. Individual thoraxes were minced with scissors and homogenized in 19 volumes of ice-cold 10 mmol l^{-1} Tris, pH 7.4, 1 mmol l^{-1} EGTA, 250 mmol l^{-1} sucrose, by a single, 10 s, low speed homogenization using a Polytron homogenizer (Brinkmann Instruments).

Rates of mitochondrial respiration in the crude thoracic homogenates were measured at 37°C in a 1.6 ml water-jacketed Gilson glass chamber, equipped with a Clark-type O_2 electrode (YSI, Yellow Springs, OH, USA). The assay buffer, consisting of 10 mmol l^{-1} Tris, pH 7.4, 1 mmol l^{-1} EGTA, 25 mmol l^{-1} KH_2PO_4, 154 mmol l^{-1} KCl, was equilibrated with room air to an oxygen content of 406 nmol O ml^{-1} (Reynafarje et al., 1985) before measurements. After the addition of 50 μl of homogenate, 10 μl of 1 mol l^{-1} pyruvate and 10 μl 1 mol l^{-1} proline were added and respiration was initiated by adding 20 μl of 40 mmol l^{-1} ADP.

Data analysis

All data are presented as species mean values ± S.D. (standard deviation), but all analyses presented below were also performed using individual data points. Species samples were randomized to reduce the effect of assay date on mean estimates of species enzyme activity, and assay date was also included in statistical analyses. The effect of body mass M_b on the different variables was tested using log-transformed data to linearise the relationship, expressed as $Y=aX^b$.

Analysis of enzyme fractional velocity was conducted by calculating the *in vivo* pathway flux rate, divided by the maximal enzyme activity (μmol min^{-1} g^{-1} thorax) ×100 to express it as a percentage (Suarez et al., 1996). Carbon dioxide production rates were converted to μmol min^{-1} g^{-1} of glycolytic flux rate, Krebs cycle rate and electron transport chain rate.

Data were also analyzed using phylogenetically independent contrasts (PIC; Felsenstein, 1985) using the PDAP module (Midford et al., 2003) included in Mesquite (Maddison and Maddison, 2004). We used the hypothesized phylogeny based on cytochrome *b* (*cyt b*) sequence from our previous work (Darveau et al., 2005) and applied two models of character evolution, using raw *cyt b* genetic distance for the gradual model, and branch lengths set to 1 for the speciational model. We ensured the contrasts were adequately standardized by plotting the absolute value of standardized independent contrasts against their S.D. (Garland et al., 1992). We also tested for branch length and topology uncertainty by performing simulations using 10 000 trees obtained from a Bayesian analysis and reported the frequency distribution of independent contrast correlation coefficient, using Mesquite.

Results

Enzyme activities

GP, TR and HK activities decline significantly with increasing body mass (Figs 1A,B and 2A). Over the 20-fold mass range, there is a 1.5-fold decrease in enzyme maximum activity in the case of both GP and TR. The smallest species, 50 mg *Euglossa sapphirina,* has average TR and GP activities of 25.7 U g^{-1} thorax and 14.7 U g^{-1} thorax, respectively, while the largest species, 1 g *Eufriesea ornata,* has TR and GP

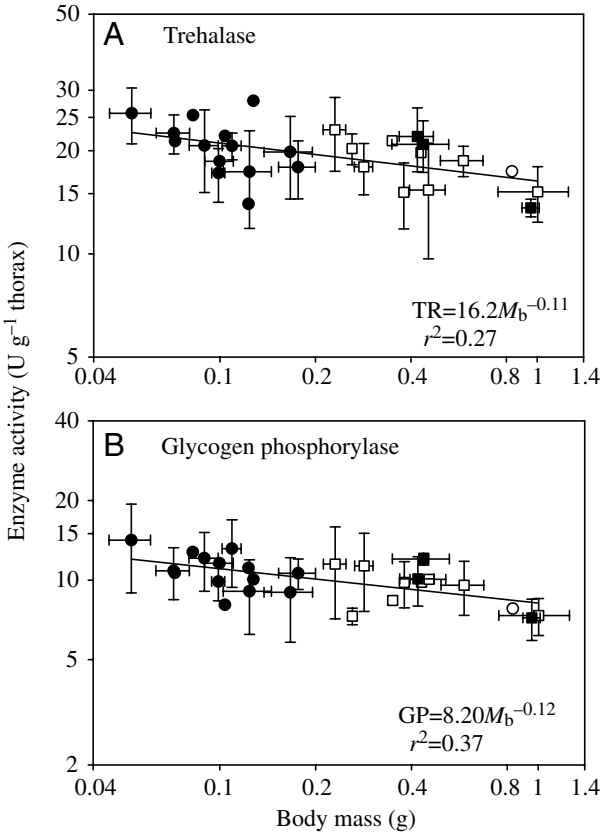

Fig. 1. Relationships between body mass and (A) trehalase (TR) and (B) glycogen phosphorylase (GP) activity measured in 27 species of orchid bees. Filled circles represent the genus *Euglossa*, open circle *Exaerete*, filled squares *Eulaema*, and open squares *Eufriesea*.

activities of 15.16 and 7.35 U g^{-1} thorax, respectively. This effect of body mass on enzyme activity is significant whether using species mean values or individual data points. HK also scales allometrically, displaying about a 2.5-fold difference in enzyme activity between the smallest and largest species and scaling as $26.9M_b^{-0.33}$ (Fig. 2A).

In contrast with the enzymes catalyzing the entry steps into glycolysis, the activities of the allosteric enzyme, PFK, are independent of body mass, averaging 108.2±18.6 U g^{-1} thorax (Fig. 2B). The activities of PGI and GPDH, enzymes that catalyze near-equilibrium reactions *in vivo* (Kashiwaya et al., 1994; Staples and Suarez, 1997), are also mass-independent, with average values of 345.6±51.0 and 616.9±133.1 U g^{-1} thorax, respectively (Fig. 2C,D).

Among mitochondrial oxidative enzymes, the Krebs cycle enzyme CS is positively related to body mass (Fig. 3A). Two clusters of data points are apparent, represented by *Euglossa* species as one group and *Exaerete*, *Eulaema* and *Eufriesea* as another. The activity of the electron transport chain enzyme, COX, is independent of species body mass (Fig. 3B). Mitochondrial respiration rates were measured in eight species of orchid bees using crude thoracic homogenates. No significant relationship between body mass and mass-specific mitochondrial respiration rate was detectable (Fig. 3C).

Flight energetics and metabolic design

We correlated the enzyme V_{max} with mass-specific metabolic rates (Darveau et al., 2005) in 14 species for which both values are available. The activities of HK, COX and GP are positively correlated with mass-specific metabolic rates (Fig. 4A–C), yielding exponents of 0.97, 0.47 and 0.36, respectively.

HK activities scale with an exponent b=–0.33, similar to that obtained for both mass-specific metabolic rate and wingbeat frequency (b=–0.31) during hovering flight (Fig. 5). Residuals obtained from body mass relationships were analyzed and yield a significant positive correlation (Fig. 6A).

In vivo pathway flux rates were calculated from mass-specific metabolic rates and used, along with V_{max} values, to estimate enzyme fractional velocities *in vivo*, i.e. $(v/V_{max})\times100$, during flight. Fig. 7 presents the relationship between body mass and fractional velocity of glycolytic and mitochondrial enzymes. This analysis reveals significant decreases in fractional velocities with increasing body mass for all enzymes except HK. In the case of HK, an average fractional velocity of 20.4±3.5% is estimated that is independent of body mass and conserved across species.

Phylogenetically independent contrasts

Independent contrast analysis was performed using a

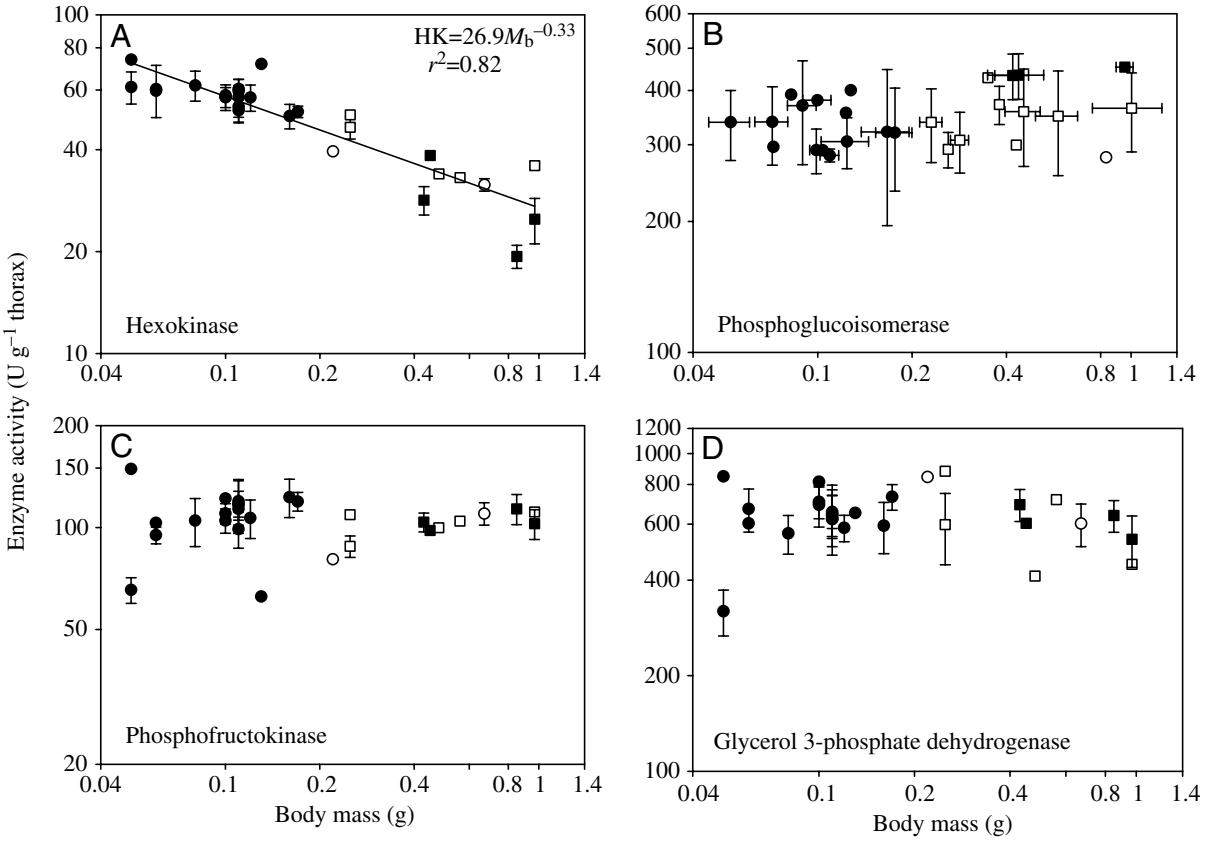

Fig. 2. Relationships between body mass and (A) hexokinase (HK), (B) phosphoglucoisomerase, (C) phosphofructokinase, and (D) glycerol 3-phosphate dehydrogenase activity measured in 28 species of orchid bees. Symbols as in Fig. 1.

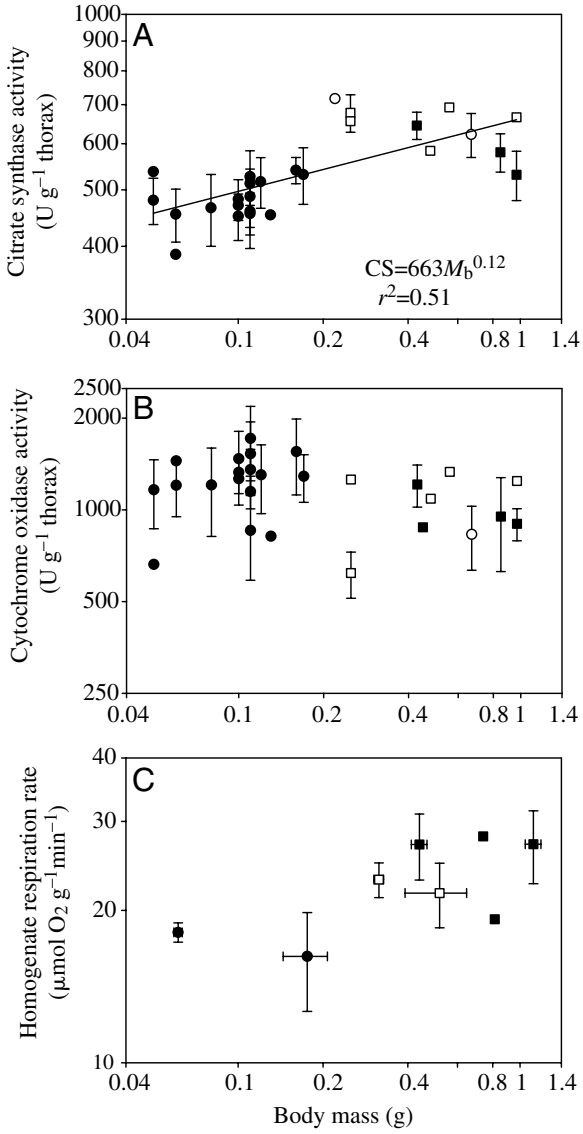

Fig. 3. Relationships between body mass and (A) citrate synthase (CS) and (B) cytochrome *c* oxidase activity measured in 28 species of orchid bees. (C) The relationship between body mass and homogenate respiration rate was measured in eight species. Symbols as in Fig. 1.

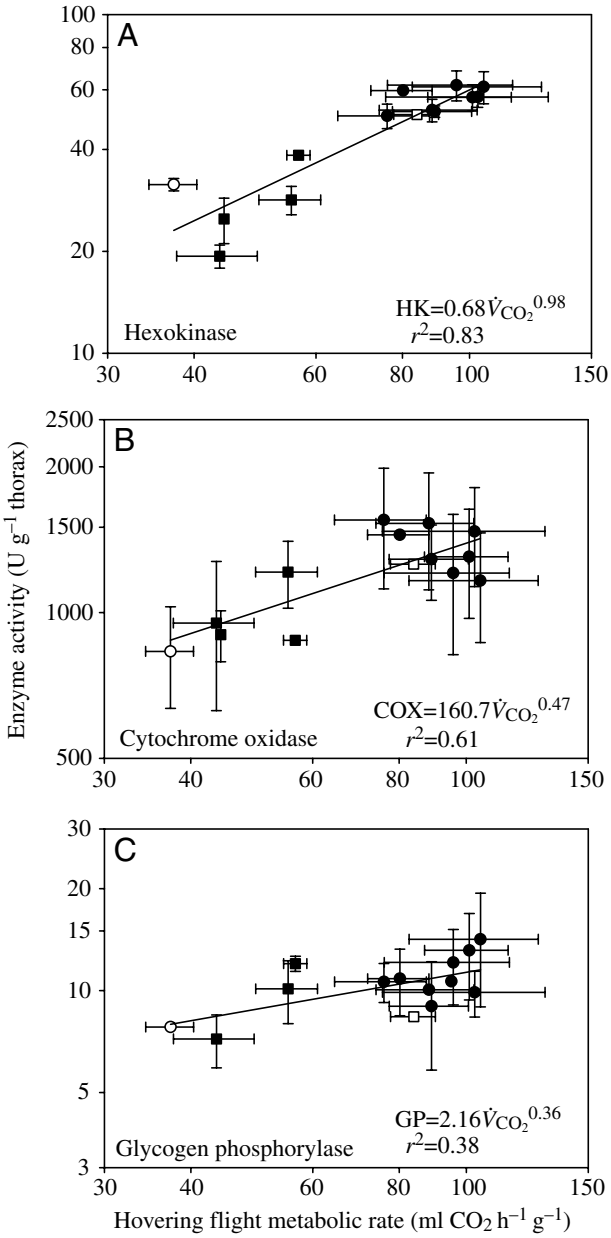

Fig. 4. Relationship between hovering flight mass-specific metabolic rate and the activity of (A) hexokinase (HK), (B) cytochrome *c* oxidase (COX) and (C) glycogen phosphorylase (GP). Symbols as in Fig. 1.

hypothetical tree based on *cyt b* gene partial sequence (Darveau et al., 2005), but to account for topology and branch length uncertainty, we implemented the analysis with distribution of correlation coefficient obtained from 10 000 trees generated from a Bayesian analysis. Fig. 8A presents the significant relationships between independent contrasts in body mass and HK activity obtained for gradual and speciational models of character evolution using the *cyt b* phylogeny. These correlation coefficients were superimposed on the distribution of correlation coefficients obtained using an array of 10 000 phylogenies (Fig. 8B). Similar analysis shows a significant effect of body mass on CS and GP, where almost all relationships are significant (Fig. 8C,D). In the case of TR, the analysis reveals that, although our proposed *cyt b* phylogeny yields a significant correlation, many other possible phylogenies do not (Fig. 8E).

The correlation between hovering flight mass-specific metabolic rates and enzyme activities were also analyzed using both our proposed *cyt b* phylogeny and 10 000 trees obtained from Bayesian analysis. For HK, the relationship was significant using all phylogenies (Fig. 9A). For COX and GP activity, most relationships were non-significant (Fig. 9B,C).

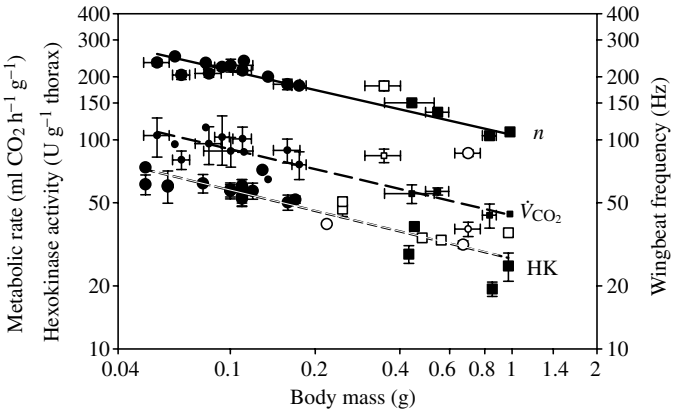

Fig. 5. Allometric scaling relationship of orchid bee hovering flight wingbeat frequency (solid line: n=106$M_b^{-0.31}$, r^2=0.86) and mass-specific metabolic rate (broken line: $\dot{V}_{CO_2}$=44$M_b^{-0.31}$, r^2=0.80), and hexokinase activity (dotted line: HK=27$M_b^{-0.33}$, r^2=0.82). Symbols as in Fig. 1.

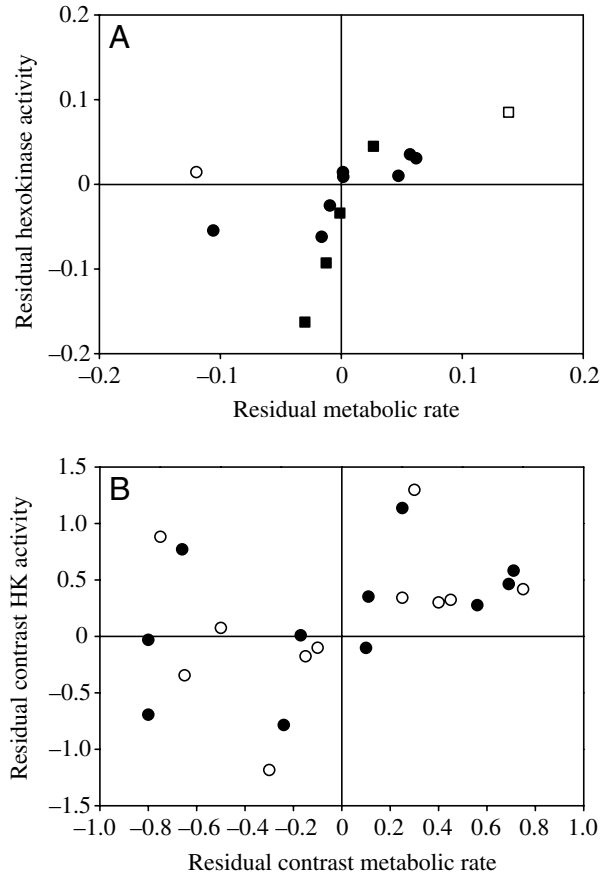

Fig. 6. (A) Correlation between hovering flight mass-specific metabolic rate and hexokinase activity residuals (r^2=0.29, $F_{1,12}$=4.85, P=0.048) obtained from the body mass relationships in Fig. 5. (B) The same relationship (non-significant in both cases) presented for independent contrast obtained from the *cyt b* phylogeny using gradual (filled circles) and speciational (open circles) models of character evolution.

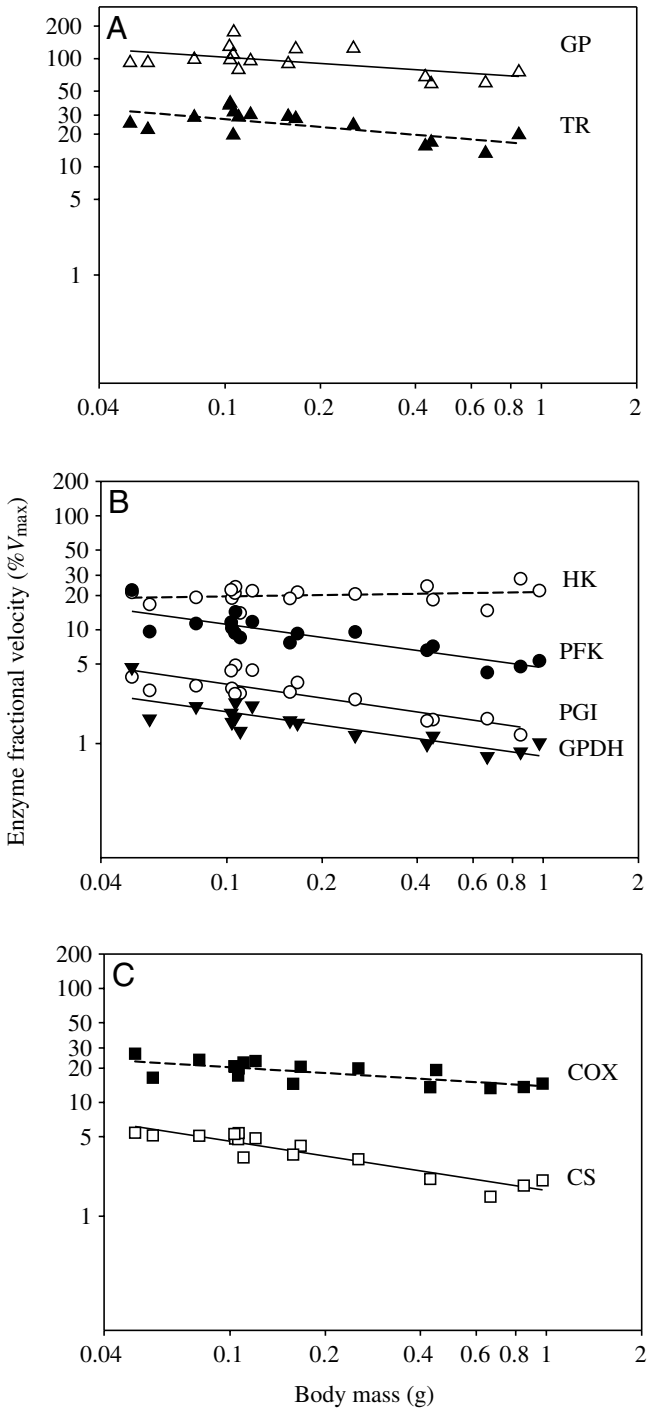

Fig. 7. Relationships between body mass and the fractional velocity (%V_{max}) of (A) glycogen phosphorylase (GP; open triangles, r^2=0.39, P<0.01) and trehalase (TR; filled triangles, r^2=0.80, P<0.001), (B) the glycolytic enzyme hexokinase (HK; open circles, P=0.65), phosphoglucoisomerase (PGI; open triangles, r^2=0.47, P<0.005), phosphofructokinase (PFK; filled circles, r^2=0.76, P<0.001) and glycerol 3-phosphate dehydrogenase (GPDH; filled triangles, r^2=0.73, P<0.001), and (C) the mitochondrial enzymes citrate synthase (CS; open squares, r^2=0.91, P<0.001) and cytochrome *c* oxidase (COX; filled squares, r^2=0.49, P<0.005).

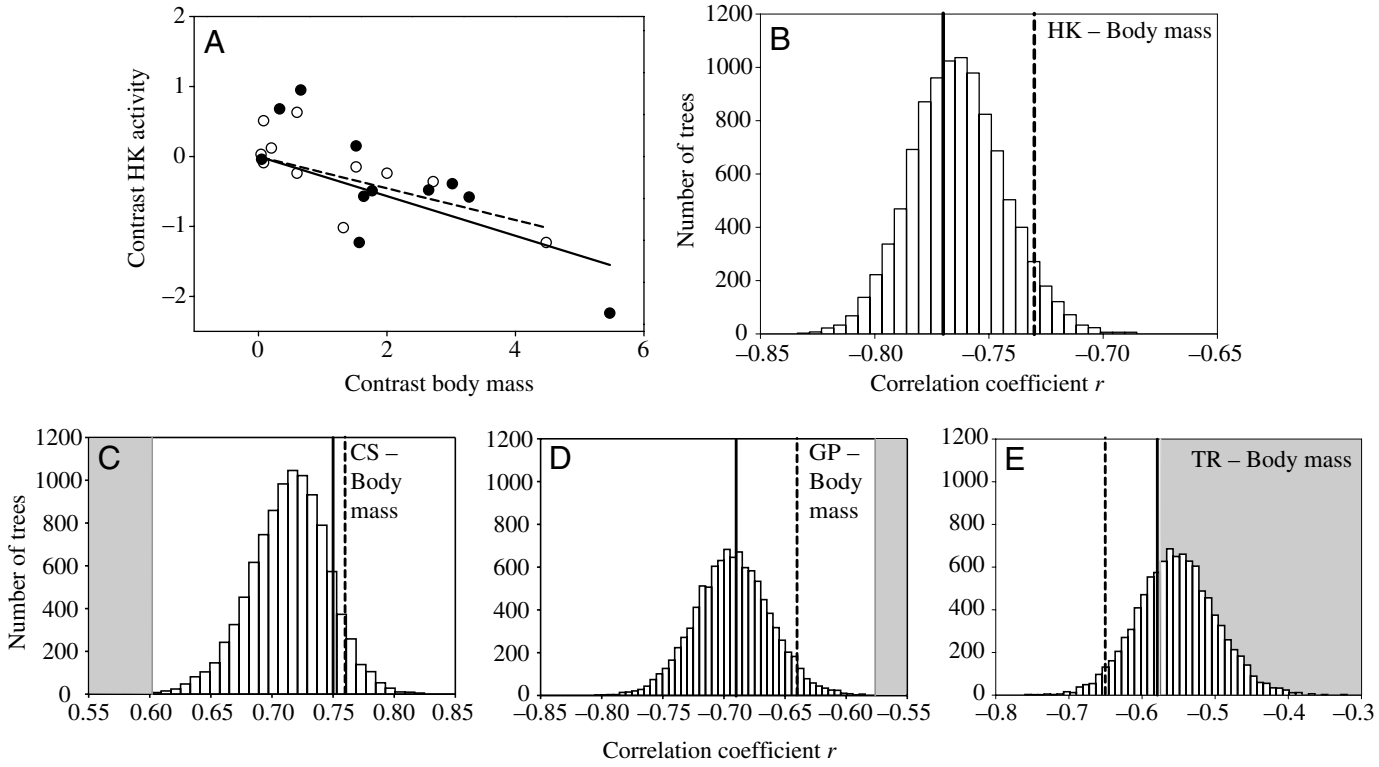

Fig. 8. Correlation coefficients between the independent contrasts in body mass and enzyme activities of (A,B) hexokinase (HK), (C) citrate synthase (CS), (D) glycogen phosphorylase (GP) and (E) trehalase (TR). Solid lines represent analyses performed using the gradual model of character evolution while broken lines represent the speciational model. The relationship between independent contrasts obtained from *cyt b* phylogeny is only presented for HK (A). The *cyt b* independent contrasts relationships are superimposed on the distribution of correlation coefficient results from analyses performed with 10 000 different trees (see Materials and methods). The correlation coefficients obtained from *cyt b* sequence information are presented for HK (gradual: r=–0.77, P=0.003; speciational: r=–0.73, P=0.007), CS (gradual: r=0.75, P=0.008; speciational: r=0.76, P=0.007), GP (gradual: r=–0.69, P=0.013; speciational: r=–0.64, P=0.025) and TR (gradual: r=–0.58, P=0.047; speciational: r=–0.65, P=0.023). The shaded areas represent non-significant relationships, given that the critical values of the correlation coefficient r for significance at the P=0.05 level (two-tailed) is 0.576 for HK, GP and TR (d.f.=10) and 0.602 for CS (d.f.=9).

Discussion

Scaling of enzyme V_{max} *values*

Allometric scaling of metabolic rate during hovering flight among orchid bees was first described by Casey et al. (1985; 1992). In a recent study (Darveau et al., 2005), we investigated the correlated evolution of wing morphology, kinematics and flight muscle energetics. In orchid bees, variation in metabolic rate during hovering is strongly related to species body mass. These body mass effects are mediated by wing size, such that wing loading and wingbeat frequency are strongly correlated after controlling for body mass effect using residual analysis. The wingbeat frequency corresponds to the flight muscle contraction frequency, which is directly related to mass-specific metabolic rate (Darveau et al., 2005). Furthermore, the relationship between wing form, kinematics and mass-specific metabolic rate is significant after controlling for phylogenetic relatedness and body mass covariation. These relationships provide the framework for our interpretation of body mass effects on physiology and metabolism.

Given the effect of body mass on metabolic rate during hovering flight in orchid bees, we hypothesized that body mass might also influence the quantitative design of flight muscle bioenergetic pathways. A direct relationship between flight muscle metabolic rate and enzyme activity would yield an approximately threefold lower activity for large orchid bee species. It is somewhat surprising that no decreases in CS or COX activities were found, given the apparent decline in mitochondrial volume densities reported by Casey et al. (1992) in orchid bees, as well as the finding of allometry in mitochondrial enzyme activities in mammalian (Emmett and Hochachka, 1981) and fish (Somero and Childress, 1980) skeletal muscles. In the study of Casey et al. (1992), mitochondrial volume ranged from about 43% in small species to 30% in large species. However, large variation was found within a given body mass, e.g. the mitochondrial volume densities of 150 mg species ranged from 35% to 43%. Such large variation in mitochondrial content may partly explain the patterns in CS and COX V_{max} values and mitochondrial respiration rates (Fig. 3A–C) reported here. Thus, unlike mass-specific metabolic rates during hovering, mitochondrial

oxidative capacities in the flight muscles do not scale allometrically.

In contrast with CS and COX, V_{max} values at the HK step decline in parallel with wingbeat frequencies and mass-specific metabolic rates (Fig. 5). The similarity in scaling relationships between the V_{max} for HK and metabolic rate suggests a functional connection between this enzymatic step and the overall rate of pathway flux. This result is particularly striking when considered in relation to previous reports of positive scaling of glycolytic enzyme V_{max} values in studies of mammals (Emmett and Hochachka, 1981), fishes (Somero and Childress, 1980), reptiles (Baldwin et al., 1995), amphibians (Miller et al., 1993) and crustaceans (Baldwin et al., 1999). Such patterns, however, occur in animals in which glycolysis is involved primarily in anaerobic ATP production. For example, in fishes, the energetic cost of 'anaerobic', burst locomotion increases with increasing body mass and positive scaling of glycolytic capacity is thought to be required to provide the energy for such activity (Somero and Childress, 1980). In contrast, glycolysis operates as part of an obligately aerobic system for ATP synthesis in insect flight muscles (Hochachka and Somero, 1973; Suarez, 2000). It seems likely that the differences between these results and ours may be partly be due to the evolution of an aerobic role by what was once an anaerobic pathway.

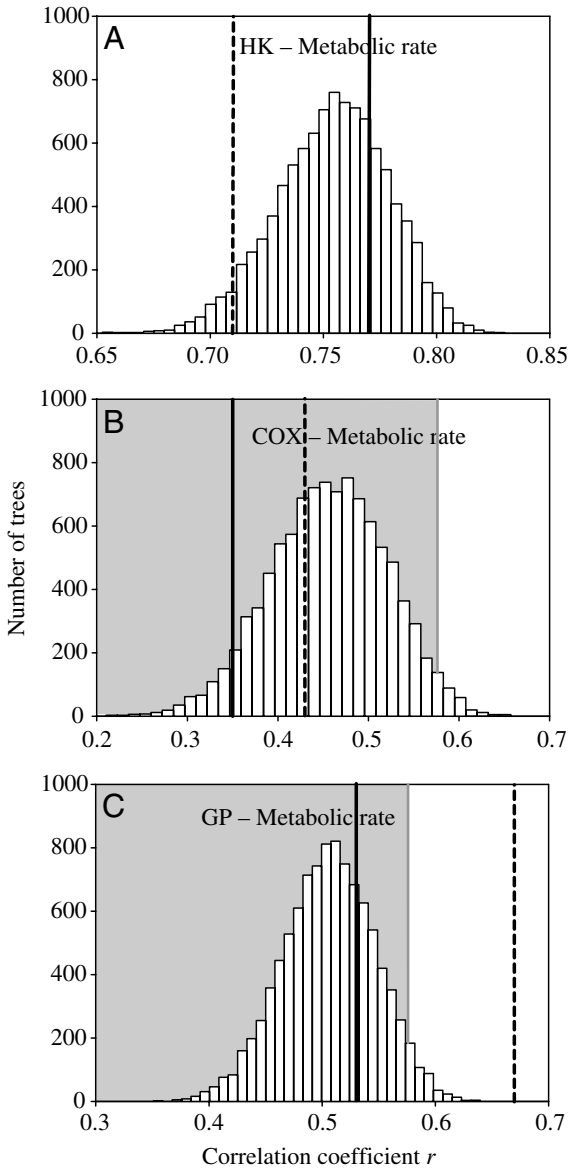

Fig. 9. Correlation coefficients between the independent contrasts in hovering flight mass-specific metabolic rate and the activity of (A) hexokinase (HK), (B) cytochrome *c* oxidase (COX) and (C) glycogen phosphorylase (GP). The distribution of correlation coefficients results from analyses performed with 10 000 different trees (see Materials and methods). The correlation coefficient obtained from *cyt b* sequence information using a gradual (solid lines) and speciational (broken lines) model of evolution are presented for HK (gradual: r=0.77, P=0.004; speciational: r=0.71, P=0.010), COX (gradual: r=0.35, P=0.27; speciational: r=0.43, P=0.16) and GP (gradual: r=0.53, P=0.07; speciational: r=0.67, P=0.02). The shaded areas represent non-significant relationships, given that the critical values of the correlation coefficient r for significance at the P=0.05 level (two-tailed) is 0.576 (d.f.=10).

Flux capacities in relation to physiological flux rates

The relationships between physiological flux rates and enzyme V_{max} values have not been evaluated within an evolutionary framework to date. In considering such data from intraspecific studies, Fell (2000) argued that the concept of rate-limiting step should be replaced with the concept of multi-site modulation of biochemical pathways. This is, of course, consistent with metabolic control theory (Fell, 1997), but it leads to the prediction that V_{max} values at multiple steps (potentially, all steps) should be co-adjusted to account for pathway flux variation (Fell, 2000). Our results show that the V_{max} values of most enzymes are independent of the variation in flux rates. Thus, among orchid bees, evolutionary changes in physiological pathway flux rates do not require the proportional changes in activity of all enzymes. Hochachka et al. (1998) considered the glycolytic pathway of red and white muscles in fish and grouped enzymatic reactions in two different categories, i.e. low and high activity enzymes, corresponding to those that catalyze reactions held far from or near-equilibrium, respectively. Hochachka et al. (1998) proposed that the largest differences in V_{max} values are found at steps catalyzed by enzymes catalyzing near-equilibrium reactions. Contrary to this, our results show that the activity of PGI does not correlate with flux rate. An alternative hypothesis proposed by Fell (2000) is that the low activity, regulatory enzymes should change the most in relation to pathway flux. This is supported by the HK results, but not by those obtained for PFK. It appears, therefore, that no simple rule allows the prediction of how V_{max} values should relate to pathway flux rates across species.

Although metabolic control theory and its application as metabolic control analysis have led to widespread abandonment of the concept of single rate-limiting steps in metabolism, examples do exist of reactions that have high flux control coefficients. Studies of cardiac muscle (Kashiwaya et al., 1994), skeletal muscle (Puigjaner et al., 1997; Fueger et al., 2004) and

myotubes (Whitesell et al., 2003) fueled strictly on glucose show that most of the control of glycolysis is shared between the HK and glucose transport. HK is known to be a regulator of glycolysis in many tissue types (Kashiwaya et al., 1994; Cardenas et al., 1998), including insect flight muscle (Saktor, 1975; Storey, 1980). Its role as a regulator of glycolytic flux in a tissue highly dependent upon exogenous glucose might explain the parallel evolution of HK V_{max}, mass-specific metabolic rate and wingbeat frequency, as well as the conservation of HK fractional velocity among orchid bees. The latter may allow the maintenance of the enzyme's regulatory role over the range of flux rates observed across species. It is tempting to speculate that the conservation of fractional velocity in this reaction allows HK to maintain its flux control coefficient, i.e. its degree of control over glycolytic flux, across species.

In a previous comparison of distantly related species (Suarez et al., 1996), it was reported that fractional velocities of the PFK reaction increase with flux rate to a maximum of about 50%. Consistent with this finding and, in contrast with the results obtained with HK, the present study shows that $\%V_{max}$ at the PFK step increases from 4% to 22%, as mass-specific metabolic rate increases and body mass declines across species. Such a decline in 'excess capacity' at this and other nonequilibrium reactions with increasing flux across species (Suarez et al., 1996) is also observed at other steps in the present interspecific study of orchid bees. Because some enzyme V_{max} values scale isometrically (PGI, PFK, GPDH, COX), or allometrically, but with exponents less than pathway flux rates (TR and GP), fractional velocities at these steps decline with increasing mass among orchid bees. Thus, species that sustain higher metabolic rates tend to have higher fractional velocities at these reactions and less excess capacity. These results beg the question of the functional significance of excess capacity at the biochemical level, and the role of evolution in selecting for or against excess enzyme protein expression. At least a partial explanation is provided by Staples and Suarez (1997) who argue that, in the case of near-equilibrium reactions, V_{max} values higher than net flux rates do not necessarily represent excess capacities. In the case of PGI in honeybee flight muscles, the high V_{max} values empirically measured are actually required for the enzyme to maintain near-equilibrium, as well as the net forward flux required for flight. In a companion paper (Suarez et al., 2005), some of the functional consequences of isometry in PGI V_{max} values in orchid bees are explored.

Broader implications

In our previous analysis of the evolution of hovering flight metabolic rate, we established a link between form and function of the flight apparatus. Here, we extend this analysis by examining the biochemical correlates of mass-related variation in form and function. By considering the results obtained from both studies, it is possible to establish the connection between the structural design of the flight apparatus and the molecular design of energy producing pathways. Over the range of body mass found in this group of bees, wing size and wing loading influence the scaling of wingbeat frequencies. Wingbeat frequencies, in turn, determine muscle power output (Pennycuick and Rezende, 1984) and, therefore, metabolic rate (Darveau et al., 2005). Authors of recent models explaining the effect of body mass on metabolic rate suggest that there is no reason to believe that the metabolic machinery (such as enzyme activity) should scale with species body mass (e.g. Banavar et al., 2002). Instead, it is proposed that metabolic rate is a consequence of limitations in the rates of material supply to cells (Banavar et al., 1999; West et al., 1999). Such a limitation has not been observed in bees at the level of the tracheal system (Harrison et al., 2001; Joos et al., 1997). Rather, it appears that the rate of energy expenditure by the contractile apparatus sets metabolic rates and the manner in which they scale. Such a conceptual model is supported by data demonstrating the correlated evolution of body mass, wing morphology, flight metabolic rate and enzymatic flux capacity. That biomechanics can adequately explain flight metabolic rate scaling in this group of bees is an alternative view to currently popular theories based on the assumption of supply limitations. Of course, the mechanisms and processes that account for the high metabolic rates during flight differ from those that contribute to resting metabolic rates (the metabolic state most often referred to in metabolic scaling studies). Nonetheless, the connection between these two metabolic states remains a subject of great interest, as studies performed on mammals (Ricklefs et al., 1996; Krosniunas and Gerstner, 2003), birds (Rezende et al., 2002) and insects (Reinhold, 1999) show a positive correlation (or, in some studies, correlated evolution) between resting and maximal metabolic rates. Our results support the suggestion (Chown et al., 2004) that, ideally, integrative analyses of body mass effects on energetics should combine mechanistic and evolutionary perspectives, as well as micro- and macrophysiological approaches.

List of symbols and abbreviations

CS	citrate synthase
COX	cytochrome *c* oxidase
cyt b	cytochrome *b* gene
[E]	enzyme concentration
GP	glycogen phosphorylase
GPDH	glycerol 3-phosphate dehydrogenase
HK	hexokinase
M_b	body mass
n	wingbeat frequency
PFK	phosphofructokinase
PGI	phosphoglucoisomerase
PIC	phylogenetically independent contrast
TR	trehalase
$\dot{V}_{CO_2}$	carbon dioxide production rate
V_{max}	enzyme maximum activity
$\%V_{max}$	enzyme fractional velocity (pathway flux/V_{max})

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