Energy metabolism in orchid bee flight muscles: carbohydrate fuels all

Raul K. Suarez[1],*, Charles-A. Darveau[2], Kenneth C. Welch, Jr. [1], Diane M. O'Brien[3],
David W. Roubik[4] and Peter W. Hochachka[2],†

[1]*Department of Ecology, Evolution and Marine Biology, University of California Santa Barbara, Santa Barbara, CA 93106-9610, USA*, [2]*Department of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4*, [3]*Biology and Wildlife Department, University of Alaska, Fairbanks, AK 99775, USA* and [4]*Smithsonian Tropical Research Institute, Balboa, Republic of Panama*

*Author for correspondence (e-mail: suarez@lifesci.ucsb.edu)
†Deceased

Summary

The widely accepted idea that bees fuel flight through the oxidation of carbohydrate is based on studies of only a few species. We tested this hypothesis as part of our research program to investigate the size-dependence of flight energetics in Panamanian orchid bees. We succeeded in measuring rates of O_2 consumption and CO_2 production *in vivo* during hovering flight, as well as maximal activities (V_{max} values) *in vitro* of key enzymes in flight muscle energy metabolism in nine species belonging to four genera. Respiratory quotients (ratios of rates of CO_2 production to O_2 consumption) in all nine species are close to 1.0. This indicates that carbohydrate is the main fuel used for flight. Trehalase, glycogen phosphorylase and hexokinase activities are sufficient to account for the glycolytic flux rates estimated from rates of CO_2 production. High activities of other glycolytic enzymes, as well as high activities of mitochondrial oxidative enzymes, are consistent with the estimated rates of carbohydrate-fueled oxidative metabolism. In contrast, hydroxyacylCoA dehydrogenase, an enzyme involved in fatty acid oxidation, was not detectable in any species. Thoracic homogenates displayed ADP-stimulated oxidition of pyruvate + proline, but did not oxidize palmitoyl L-carnitine + proline as substrates. A metabolic map, based on data reported herein and information from the literature, is presented. The evidence available supports the hypothesis that carbohydrate serves as the main fuel for flight in bees.

Key words: respiratory quotient, hovering flight, Euglossine bee, enzymes, glycolysis, flux, carbohydrate.

Introduction

Bees have served as experimental models in studies of many aspects of insect flight that include wing kinematics, muscle biomechanics, thermoregulation, allometric scaling and metabolic biochemistry. Honeybees (*Apis mellifera*), in particular, are convenient to use, display extremely high metabolic rates during flight, and have been used to address biochemical questions concerning fuel use (Blatt and Roces, 2001; Gmeinbauer and Crailsheim, 1993), pathway design and metabolic flux rates (Suarez, 2000), as well as mitochondrial function during flight (Suarez et al., 2000). Honeybees fuel their flight muscles with hexose sugars (Blatt and Roces, 2001; Gmeinbauer and Crailsheim, 1993; Suarez, 2000) and possess high enzymatic flux capacities (Staples and Suarez, 1997; Suarez et al., 1996). During flight, metabolic flux rates are so high that some enzymes operate close to their maximal capacities (Staples and Suarez, 1997; Suarez et al., 1996, 2000). The mitochondrial volume densities and cristae surface densities required to support high rates of aerobic ATP synthesis are close to theoretical upper limits in locomotory muscles (Suarez, 1996), and electron transfer rates between mitochondrial respiratory chain enzymes are the highest ever measured in animals (Suarez et al., 1999, 2000).

A question that arises is whether the picture that has emerged concerning the design and function of pathways of energy metabolism in honeybees also applies to other species of bees. Although some insect species appear to fuel flight with carbohydrate only, others can use both carbohydrate and fat, while still other species rely primarily on amino acids (Storey, 1985). The evolution of metabolic fuel choice in insect flight muscles is not well understood; however, much of the interspecific variation in this appears to be related to factors such as diet, foraging ecology and dispersal. More than three decades ago, Crabtree and Newsholme (1972a,b) conducted comparative biochemical studies that have since become part of the foundation for our understanding of the diversity of fuel use in flying insects. On the basis of maximum enzyme activities they reported in honeybees and bumblebees, RQ values of 1.0 reported by other investigators (e.g. Rothe and

Nachtigall, 1989), and measurements of hemolymph sugars (Blatt and Roces, 2001; Gmeinbauer and Crailsheim, 1993), it is now assumed that, in general, bees use carbohydrates to fuel flight. These, as well as the greater sensitivity of CO_2 analyzers compared with O_2 analyzers, have led researchers (including ourselves) in recent years to measure metabolic rates only as CO_2 production rates ($\dot{V}_{CO_2}$), assuming that these equal O_2 consumption rates ($\dot{V}_{O_2}$). The great diversity in metabolic organization known amongst insects as well as the limited number of species of bees subjected to detailed study warrant caution in this regard.

Inspired by the pioneering work of Casey, Ellington and colleagues (Casey and Ellington, 1990; Casey et al., 1992, 1985), we embarked upon studies of the allometric scaling of energy expenditure during flight (Darveau et al., 2005a) and the biochemistry underlying such scaling (Darveau et al., 2005b; Suarez et al., 2005) in Panamanian orchid bees. Orchid bees consist of more than 190 species belonging to five genera and range from about 50 to >1000 mg in body mass. Given the diversity in fuel use among flying insects, we considered it essential, as part of our research program, to examine enzymatic capacities and rates of fuel oxidation during flight within this clade to test the hypothesis that carbohydrate oxidation serves as the main source of energy during flight. The interspecific data reported herein, consisting of enzyme V_{max} values measured *in vitro*, as well as metabolic rates and RQ ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) values estimated *in vivo*, include nine species belonging to four of the five extant genera. They provide support for the hypothesis that orchid bee flight muscles have evolved to specialize in the use of carbohydrate oxidation to supply their energetic requirements.

Materials and methods

Orchid bee collection

Orchid bees (for details, see Table 1) were captured on Barro Colorado Island at the Smithsonian Tropical Research Institution in Panama. Only males were used, as these could be attracted to and captured after landing on filter 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).

Respirometry measurements

Measurements of metabolic rate during flight were conducted immediately after capture to minimize time-dependent changes in motivation. Smaller species, up to 400 mg in body mass, were flown in a 0.5 l flask with sidearm, while a similar flask of 1 l capacity was used for larger species. Air was drawn into the flasks through perforated rubber stoppers and out through the sidearms through Tygon® tubing at a rate of $1.5\,l\,min^{-1}$ by a FOX flow-through field respirometry system equipped with a Sable Systems (Henderson, NV, USA) CA-2A CO_2 analyzer. The CO_2 analyzer was calibrated daily using a 5.03% CO_2 span gas. Flight durations of 2–3 min, facilitated by slight shaking and tilting of the flasks when bees attempted to land, were sufficient to yield steady-state rates of O_2 consumption and CO_2 production. Data acquisition and analysis were performed using Datacan (Sable Systems). Because of the lower limit to the sensitivity of our O_2 analyzer, individuals of less than 100 mg body mass often yielded $\dot{V}_{O_2}$ values that were difficult to reproduce. Therefore, all $\dot{V}_{O_2}$ values from individuals of <100 mg mass were discarded.

Enzyme activities

Other 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

Table 1. *Body masses, rates of whole-body O_2 consumption, CO_2 production and respiratory quotients in nine species of orchid bees*

Species	Mass (g)	N	$\dot{V}_{O_2}$ (ml $O_2\,g^{-1}\,h^{-1}$)	$\dot{V}_{CO_2}$ (ml $O_2\,g^{-1}\,h^{-1}$)	RQ ($\dot{V}_{CO_2}/\dot{V}_{O_2}$)	Glycolytic rate (μmol $g^{-1}\,min^{-1}$)
Eg. tridentata	0.1076±0.0104	4	102.65±19.93	102.38±17.50	1.00±0.06	12.70±2.17
Eg. cognata	0.1633±0.0221	3	89.21±25.09	84.27±13.33	0.97±0.12	10.45±1.65
Eg. imperialis	0.1766±0.0087	4	95.36±41.44	80.30±13.45	0.91±0.23	9.96±1.67
El. nigrita	0.4179±0.1133	3	58.11±11.55	56.84±5.98	0.99±0.09	7.05±0.74
El. cingulata	0.5626±0.0447	3	55.88±4.18	57.37±1.97	1.03±0.07	7.11±0.24
El. meriana	0.9252±0.0409	4	49.24±6.74	44.48±4.57	0.91±0.14	5.52±0.57
El. bombiformis	0.9828±0.0787	3	44.90±3.58	44.18±4.49	0.98±0.05	5.48±0.56
Ex. frontalis	0.6773±0.0496	6	38.84±7.20	37.23±3.22	0.97±0.12	4.62±0.40
Ef. pulchra	0.3510±0.0506	5	88.31±13.99	83.97±6.23	0.96±0.09	10.41±0.77

$\dot{V}_{CO_2}$, rate of CO_2 production; $\dot{V}_{O_2}$, rate of O_2 consumption; RQ, respiratory quotient.

Abbreviated genus names are *Euglossa* (*Eg.*), *Eulaema* (*El.*), *Eufriesia* (*Ef.*) and *Exaerete* (*Ex.*).

Glycolytic flux rates are calculated from $\dot{V}_{CO_2}$ values and represent rates of hexose sugar utilization, expressed in μmol g^{-1} thorax min g^{-1}. Data are means ± S.D.; *N* is the sample size.

homogenization buffer used on samples used 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., 1986a). 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 Micro Ultrasonic Cell Disrupter (Kontes, 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 Cole-Parmer 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 glycerol 3-phosphate dehydrogenase. GPDH: 50 mmol l^{-1} imidazol, pH 7.0, 1 mmol l^{-1} dihydroxyacetonephosphate (omitted for control), 0.15 mmol l^{-1} NADH. CS: 50 mmol l^{-1} Tris-HCl, pH 8.0, 0.5 mmol l^{-1} oxaloacetate (omitted for 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 for 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.

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 often 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, using a single, 10 s, low speed homogenization using a Polytron homogenizer (Brinkman Instruments) with a small tip.

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$_2$PO$_4$, 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 (or 10 μl 5 mmol l^{-1} palmitoyl L-carnitine) and 10 μl 1 mol l^{-1} proline were added and respiration was initiated by adding 20 μl 40 mmol l^{-1} ADP.

Results and discussion

We succeeded in collecting a complete set of $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and enzyme V_{max} values from a total of nine species ranging in mean body mass from about 0.1 to 1.0 g (Tables 1, 2 and 3).

Table 2. *Body masses and* V*~max~ values for glycogen phosphorylase (GP), trehalase (TR) and phosphoglucoisomerase (PGI)*

Species	Body mass	N	TR	GP	PGI
Eg. tridentata	0.1091±0.0075	3	20.65±1.85	13.15±3.76	283.63±9.61
Eg. cognata	0.1665±0.0289	4	23.09±2.10	10.85±1.55	400.32±27.83
Eg. imperialis	0.1763±0.0234	4	16.36±5.61	10.70±1.36	334.08±89.81
El. nigrita	0.4191±0.0514	6	22.46±4.93	9.79±2.13	435.84±56.48
El. cingulata	0.4373±0.0888	3	20.87±3.58	12.02±0.62	433.60±52.05
El. meriana	0.9553	2	13.63	7.35	452.51
Ex. frontalis	0.8330	1	17.42	7.80	280.71
Ef. pulchra	0.3493	1	21.40	8.38	427.62

Genus names are abbreviated as in Table 1.

N is the sample size; data are means ± S.D. except when $N<3$.

Enzyme activities represent maximum rates of substrate conversion to product at 37°C, expressed in μmol g^{-1} thorax min g^{-1}.

Although it was possible to obtain reliable $\dot{V}_{CO_2}$ data from smaller species (Darveau et al., 2005a), discarding $\dot{V}_{O_2}$ values obtained from species weighing <100 mg limited the number of species that could be included. Nevertheless, RQ data were obtained from species spanning a tenfold range in body mass and include representatives of all but one genus.

The biochemical data (Tables 2 and 3) reveal high enzymatic capacities for carbohydrate oxidation. High citrate synthase (a Krebs cycle enzyme) and cytochrome oxidase (a respiratory chain enzyme) activities are not unexpected, given the high rates of aerobic metabolism required to support flight and the high mitochondrial volume densities previously reported (Casey et al., 1992). High hexokinase activities, indicating high capacities for hexose sugar phosphorylation, range from values of about half to up to twofold higher than those found in honeybees (Suarez et al., 1996). GPDH, an enzyme catalyzing a near-equilibrium reaction required for the maintenance of high cytosolic NAD$^+$/NADH (as part of the glycerol 3-phosphate shuttle; Crabtree and Newsholme, 1975; Sacktor, 1976), occurs at higher V_{max} values than all the other glycolytic enzymes measured. Also consistent with high glycolytic capacities are the high V_{max} values for PGI, a glycolytic enzyme catalyzing a near-equilibrium reaction (Staples and

Suarez, 1997), and PFK, an allosteric enzyme catalyzing a nonequilibrium reaction (Wegener et al., 1986a,b).

Hydroxyacyl-CoA dehydrogenase, an enzyme involved in fatty acid oxidation (Crabtree and Newsholme, 1975), was not detectable in thoracic extracts of any species. Homogenates prepared for measurement of mitochondrial O$_2$ consumption displayed the capacity to oxidize pyruvate, but not palmitoyl L-carnitine (Fig. 1). Proline was included in assays when testing for pyruvate or palmitoyl L-carnitine oxidation, based on the results of preliminary studies (R. K. Suarez, unpublished observations) with mitochondria isolated from honeybee flight muscles. These revealed that, although pyruvate or proline alone are not oxidized at significant rates, pyruvate plus proline support high rates of coupled, state 3 (i.e. ADP-stimulated) respiration. Such results are interpreted in terms of proline having an anaplerotic role (i.e. the augmentation of Krebs cycle intermediates) required for high rates of Krebs cycle activity (Sacktor and Childress, 1967). Malate, however, is unable to serve such a role (R. K. Suarez, unpublished observations), in contrast with mitochondria isolated from locust flight muscles (Suarez and Moyes, 1992), and so was not used in the studies reported here. The rates measured in 21 individuals of eight species (*Eg. sappharina*, *Eg. imperialis*, *Ef. chrysopyga*, *Ef.*
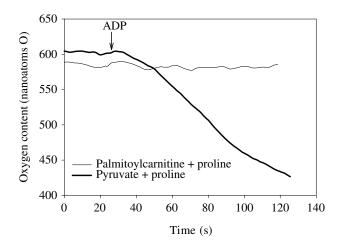
Table 3. *Body masses and* V*~max~ values for hexokinase (HK), phosphofructokinase (PFK), glycerol 3-phosphate dehydrogenase (GPDH), citrate synthase (CS) and cytochrome oxidase (COX)*

Species	Body mass	N	HK	PFK	GPDH	CS	COX
Eg. tridentata	0.1203	7	57.01±5.06	106.7±13.9	583.8±55.6	516.4±51.6	1303±330
Eg. cognata	0.1673	5	51.77±2.04	119.5±7.6	731.3±67.6	531.4±59.1	1288±228
Eg. imperialis	0.1583	6	50.24±4.20	123.0±15.9	593.3±109.2	540.2±27.9	1553±433
El. nigrita	0.4295	5	28.38±2.75	103.8±6.7	691.1±79.6	644.7±34.7	1212±191
El. cingulata	0.4485	1	38.43	98.1	603.4		876
El. bombiformis	0.9730	6	24.91±3.84	102.7±10.4	537.6±98.9	530.7±51.7	900±108
El. meriana	0.8486	5	19.35±1.52	113.6±11.6	638.5±72.9	580.1±43.8	951±323
Ex. frontalis	0.6660	5	31.49±1.32	110.0±8.4	602.0±92.1	622.2±53.8	831±197
Ef. pulchra	0.2544	1	50.68	109.2	880.0	655.8	1257

Genus names are abbreviated as in Table 1.

N is the sample size; data are means ± S.D. except when $N<3$.

Enzyme activities represent maximum rates of substrate conversion to product at 37°C, expressed in μmol g^{-1} thorax min^{-1}.

Fig. 1. Oxygen consumption *in vitro* by thoracic homogenate from a 1.11 g *Eulaema bombiformis*. 50 μl samples were injected into 1.60 ml of assay medium. 20 μl of 40 mmol l^{-1} ADP was injected where indicated. No stimulation of respiration by ADP is observed when substrates provided are 0.03 mmol l^{-1} palmitoyl L-carnitine + 6 mmol l^{-1} proline. Injection of ADP results in stimulation of respiration when 6 mmol l^{-1} pyruvate + 6 mmol l^{-1} proline are provided as substrates. The slope of the linear part of the trace after ADP injection yields a rate of 144 nanoatoms O min^{-1}. Note the break at the end of the ADP-stimulated (state 3) rate and the beginning of a lower (state 4) rate, as would normally be expected after depletion of ADP. Because our protocol makes use of crude homogenates, the state 4 rate is still high relative to the state 4 rates obtainable with isolated, well-coupled, mitochondria. In addition, such transitions between state 3 and state 4 rates were not always observed. This is to be expected, given the high contaminating ATPase activities found in tissue homogenates. This limitation in our protocol precluded meaningful estimates of respiratory control (state 3/state 4) and ADP/O ratios.

schmidtiana, El. bombiformis, El. nigrita, El. cingulata, El. meriana) were mass-independent and averaged 22.54 ± 5.02 μmol O_2 g^{-1} thorax min^{-1} (means ± S.D.).

The *in vitro* data are consistent with RQ values obtained during flight that do not differ significantly from 1.0 (Table 1), indicating that carbohydrate oxidation provides the energy for flight. Given the use of carbohydrate as the main fuel, it is possible to estimate the glycolytic flux rates required to supply pyruvate to the flight muscle mitochondria. Crabtree and Newsholme (1972b) reported glycogen phosphorylase activities in honeybee flight muscles that are insufficient to account for glycolytic flux rates during flight. In contrast, maximal capacities for glycogen breakdown are sufficient to provide hexose phosphate at the rates required to sustain flight in orchid bees (Tables 1 and 2). It has been proposed that thoracic

glycogen might be used to buffer hexose phosphate concentrations during transitions between rest and flight (Crabtree and Newsholme, 1975; Sacktor, 1976) or to extend flight range (Harrison, 1986) in honeybees. That glycogen phosphorylase activities sufficient to account for glycolytic rates are found in orchid bees suggests that glycogen may play a greater role in fueling flight in these species than in honeybees. On the other hand, trehalase activities are about 1.5- to twofold greater than those of glycogen phosphorylase. In honeybees, Blatt and Roces (2001) estimated sugar flux rates between proventriculus, fat bodies and flight muscles that are
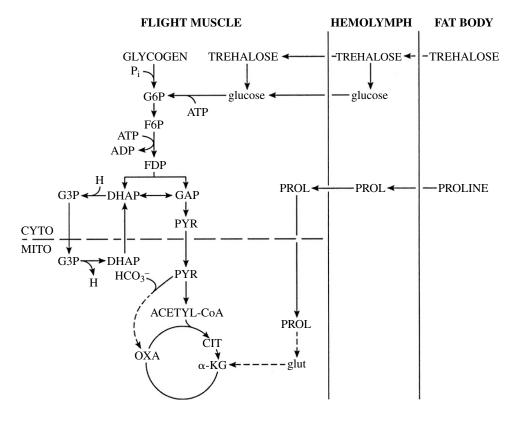

Fig. 2. Proposed pathways for substrate oxidation in bees, showing roles played by fat body, hemolymph, cytoplasmic and mitochondrial reactions. Not all reactions are included and details concerning individual reactions are left out to emphasize the main routes of carbon flow, the glycerol 3-phosphate (G3P) shuttle, and anaplerotic (pyruvate carboxylase and proline oxidation) reactions. Abbreviations are as follows: P_i, inorganic phosphate, G6P, glucose 6-phosphate, F6P, fructose 6-phosphate, FDP, fructose 1,6-diphosphate, DHAP, dihydroxyacetone phosphate, GAP, glyceraldehyde 3-phosphate, pyr, pyruvate, G3P, glycerol 3-phosphate, H, reducing equivalent, OXA, oxaloacetate, CIT, citrate, αKG, alpha-ketoglutarate, glut, glutamate, PROL, proline, CYTO, cytoplasmic compartment, MITO, mitochondrial matrix.

sufficient to support flight. It is likely, given the high enzymatic capacities for trehalose and glucose metabolism in orchid bees, as well as the high rate at which limited glycogen stores would be depleted, that hexose sugars from the hemolymph serve as the predominant fuels for flight in these species as well.

The great consistency between the results presented herein and those obtained with honeybees warrant the proposal of a hypothetical metabolic scheme that combines features based on the results of various studies (Fig. 2). The anaplerotic role of proline in honeybees, also shown in Fig. 2, is further supported by results showing its depletion from hemolymph during flight (Micheu et al., 2000). Another reaction with such a role is that catalyzed by pyruvate carboxylase, an enzyme found at high activities in honeybee flight muscles (Crabtree et al., 1972; Tu and Hagedorn, 1992). This intramitochondrial enzyme catalyzes the carboxylation of pyruvate to oxaloacetate and is allosterically activated by acetylCoA. The diagram also shows fat body as the source of trehalose (Becker et al., 1996), and trehalase activity occuring in both intracellular as well as extracellular compartments (Becker et al., 1996; Brandt and Huber, 1979).

In conclusion, apart from the anaplerotic role played by proline, carbohydrate oxidation predominates as the major source of energy for flight in bees. We have found no evidence to indicate that fatty acid oxidation plays a role in fueling flight. These findings provide added justification for the measurement of $\dot{V}_{CO_2}$ values and the assumption that RQ=1.0 in studies of flight energetics in bees. Given the use of fats by many other taxa of flying insects, one is led to ask why the biochemical machinery required to use such an energy-rich substrate has apparently been discarded by this lineage. Unlike the honeybees, orchid bees do not store honey in hives and do not display clear eusociality. Observed behaviour ranges from being solitary, as observed in some *Eufriesea* and *Euglossa*, to being weakly social in *Eulaema*, to 'threshold eusociality' in *Euglossa* (Roubik and Hanson, 2004). Despite this range of behaviours, reliance upon carbohydrate oxidation by flight muscles is a feature common to all bee species studied, thus far. The wasps from which bees evolved are represented by extant relatives that prey on other insects. However, flight metabolism in wasps has not been examined in detail. Clearly, the evolution of fuel use in flying insects is a subject that warrants further investigation.

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