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MITOCHONDRIAL FUNCTION IN FLYING HONEYBEES (*APIS MELLIFERA*): RESPIRATORY CHAIN ENZYMES AND ELECTRON FLOW FROM COMPLEX III TO OXYGEN

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Summary

The biochemical bases for the high mass-specific metabolic rates of flying insects remain poorly understood. To gain insights into mitochondrial function during flight, metabolic rates of individual flying honeybees were measured using respirometry, and their thoracic muscles were fixed for electron microscopy. Mitochondrial volume densities and cristae surface densities, combined with biochemical data concerning cytochrome content per unit mass, were used to estimate respiratory chain enzyme densities per unit cristae surface area. Despite the high content of respiratory enzymes per unit muscle mass, these are accommodated by abundant mitochondria and high cristae surface densities such that enzyme densities per unit cristae surface area are similar to those found in mammalian muscle and liver. These results support the idea that a unit area of mitochondrial inner membrane constitutes an invariant structural unit. Rates of O_2 consumption per unit cristae surface area are much higher than those estimated in mammals as a consequence of higher enzyme turnover rates (electron transfer rates per enzyme molecule) during flight. Cytochrome *c* oxidase, in particular, operates close to its maximum catalytic capacity (k_{cat}). Thus, high flux rates are achieved *via* (i) high respiratory enzyme content per unit muscle mass and (ii) the operation of these enzymes at high fractional velocities.

Key words: honeybee, *Apis mellifera*, respiratory chain enzyme, electron flow, complex III, flight, metabolic rate, oxygen consumption, respirometry, mitochondria, cytochrome.

Introduction

Flying insects achieve the highest mass-specific rates of aerobic metabolism in the Animal Kingdom (Sacktor, 1976). Although insects have served as useful models in studies concerning the regulation of energy metabolism (e.g. Hansford, 1974; Rowan and Newsholme, 1979; Wegener et al., 1991), the issue of how such high rates of metabolism are achieved remains unresolved. Physiologists have known for decades that insects possess high capacities for O_2 transport through their tracheal systems (Weis-Fogh, 1964). Biochemists have long been aware of high enzymatic flux capacities in catabolic pathways (e.g. Crabtree and Newsholme, 1975) and of the abundance of mitochondria (e.g. Smith, 1963) in insect flight muscles. However, for a more quantitative, mechanistic understanding of how flying insects achieve their phenomenal rates of respiration, data concerning the rates at which metabolic enzymes and mitochondria function *in vivo* are required.

We have undertaken a series of studies comparing biochemical flux capacities with physiological flux rates during flight in honeybees (*Apis mellifera*). The mass-specific metabolic rates obtained for flying honeybees are approximately three times greater than those measured in hovering hummingbirds (e.g. Bartholomew and Lighton, 1986; Suarez, 1992) and 30 times greater than those achieved by human athletes exercising at $\dot{V}_{O_2max}$ (e.g. Blomstrand et al., 1986). We found that flight muscle hexokinase operates close to its maximal velocity (V_{max}) while phosphofructokinase works at approximately half-maximal velocity in flying honeybees (Suarez et al., 1996). Although the V_{max} value for phosphoglucoisomerase is 20 times higher than maximal glycolytic flux rates, when the near-equilibrium position of this reaction *in vivo* is considered, up to 95 % of this flux capacity is required during flight (Staples and Suarez, 1997). Analogous studies of mitochondrial respiration pose a greater challenge because of the complexity of the respiratory enzymes and their localization on the inner mitochondrial membranes (Saraste, 1999). In euglossine bees, Casey et al. (1992) estimated higher mitochondrial volume densities, cristae surface densities and

rates of O_2 consumption per unit mitochondrial volume than those reported in mammals (Hoppeler and Lindstedt, 1985). The higher mitochondrial respiration rates indicate that the high respiration rates displayed by flying insects are not simply the result of proportionately greater mitochondrial content in their flight muscles. This led Casey et al. (1992) to recommend that biochemical studies be conducted to elucidate the mechanistic bases for their findings.

In the present study, we measured the metabolic rates of individual honeybee workers during flight. Each thorax was then fixed for electron microscopy, and flight muscle mitochondrial volume and cristae surface densities were estimated. Data concerning cytochrome content per unit mass (Suarez et al., 1999) are used to estimate respiratory chain enzyme content per unit cristae surface area. Electron flux rates per respiratory chain enzyme molecule, as well as the fractional velocity (v/V_{max}) of cytochrome *c* oxidase, are estimated in flying honeybees.

Materials and methods

Respirometry

Honeybees (*Apis mellifera*) were obtained and respirometry was performed as described previously (Suarez et al., 1999). Briefly, workers were caught with perforated plastic bags while exiting their hive to forage. Immediately after capture, individual bees were released into a 0.5 l flow-through respirometry chamber. Flight was induced by hitting the chamber with rubber stoppers attached to the end of a stick. $\dot{V}_{CO_2}$ was measured at 22 °C using a Sable Systems respirometry system (Henderson, NV, USA) as described previously (Suarez et al., 1999).

Electron microscopy

Thoraces from eight honeybees were cut in half mid-sagittally and immersed in glutaraldehyde fixative (6.25 % solution in 0.1 mol l^{-1} sodium cacodylate buffer; total osmolarity of the fixative 1100 mosmol l^{-1}; pH 7.4) for at least 24 h. Two slabs (approximately 4 mm long, 2 mm wide and 1 mm thick) of the longitudinal muscle were taken parallel to the midline on either side and completely cut into small blocks approximately 1 mm×1 mm×2 mm. They were processed for electron microscopy as described previously (Mathieu-Costello, 1987), and ultrathin sections (50–70 nm) were cut transversely to the muscle fiber axis in two blocks from each bee. They were contrasted with uranyl acetate and bismuth subnitrate (Riva, 1974), and electron micrographs for morphometry were taken on 70 mm film using a Zeiss l0 electron microscope. Morphometric measurements of the volume density of mitochondria and the surface density of inner and outer mitochondrial membranes were performed as described previously (Hoppeler et al., 1981; Mathieu et al., 1981). Briefly, the volume densities of mitochondria and myofibrils were estimated by point-counting at a final magnification of 23 000× on 20 fields obtained by systematic random sampling in one ultra-thin transverse section from each of the two blocks; in total, 40 fields per sample. The surface density of inner and outer mitochondrial membranes per volume of mitochondria was estimated by intersection-counting at a final magnification of 125 000× on 20 fields obtained by systematic random sampling of the same ultra-thin sections used to estimate volume densities. Contact prints of the 70 mm films were projected onto a screen fitted with a 144-point quadratic grid of lines for both volume and surface density measurements, and both horizontal and vertical lines were used to count intersections.

Calculations

Measured $\dot{V}_{CO_2}$ values were considered equal to $\dot{V}_{O_2}$ because the respiratory quotient ($=\dot{V}_{CO_2}/\dot{V}_{O_2}$) is 1.0 during flight (Rothe and Nachtigall, 1989). To estimate thoracic and muscle metabolic rates, it was assumed that muscle mass is 75 % of thorax mass (Nachtigall et al., 1995), that muscle density is 1.06 g cm^{-3} and that fiber volume is 90 % of muscle volume (Schwerzmann et al., 1989).

The number of respiratory chain enzyme complexes per square micrometer of cristae surface area was estimated according to the method of Schwerzmann et al. (1986, 1989). Complex III (cytochrome bc_1), cytochrome *c* and complex IV (cytochrome *c* oxidase) contents per gram of thorax were measured by difference spectroscopy and have been published previously (Suarez et al., 1999). Enzyme densities per unit cristae surface area were estimated by dividing enzyme content per gram by cristae surface area per gram. Enzyme turnover rates were estimated by converting the mean thoracic rate of O_2 consumption to electron flux rate, given 4 electrons accepted per O_2 consumed (Nicholls and Ferguson, 1992), and then dividing the electron flux rate per gram by enzyme content per gram as before (Suarez et al., 1999).

Results and discussion

Respirometry

Individual and mass-specific metabolic rates during agitated flight (Table 1) are similar to those reported previously by ourselves (Joos et al., 1996; Suarez et al., 1996, 1999) and others (Rothe and Nachtigall, 1989; Wolf et al., 1989; Harrison and Hall, 1993). In a recent study (Suarez et al., 1999), we showed that metabolic rates during agitated flight are significantly higher than those achieved during hovering. Thus, for the purpose of estimating the highest metabolic rates obtainable with unladen bees at ambient P_{O_2} and pressure, the technique we employed is appropriate.

Morphometry and mitochondrial respiration rates

In highly aerobic muscles such as those used for insect flight, mitochondria synthesize most of the ATP required during contraction by actomyosin ATPase, Ca^{2+}-ATPase and Na^+/K^+-ATPase (Suarez, 1996). Because asynchronous flight muscles, such as those found in bees, possess a poorly developed sarcoplasmic reticulum (Ellington, 1985), it is thought that most of the ATP hydrolyzed is used by actomyosin ATPase

Table 1. *Mass and metabolic rate data*

Body mass (mg)	82.61±5.99
Thorax mass (mg)	28.55±0.44
Metabolic rate	
Individual (ml O_2 h^{-1})	9.13±0.25
Mass-specific (ml O_2 g^{-1} h^{-1})	114.8±10.6
Thoracic (ml O_2 g^{-1} h^{-1})	320.2±9.1
Muscle (ml O_2 cm^{-3} min^{-1})	8.38±0.24

Values are means ± S.E.M. of data from eight individuals.

Table 2. *Morphometric measurements and rates of mitochondrial respiration* in vivo

Volume density (% fiber volume)	
Myofibril	53.8±0.5
Mitochondria	43.0±0.5
Surface density (m^2 cm^{-3})	
Outer membrane	3.0±0.1
Inner membrane	48.0±2.4
Mitochondrial respiration rate	
Total volume (ml O_2 cm^{-3} min^{-1})	19.49±0.49
Cristae surface area (µl O_2 m^{-2} min^{-1})	414.1±27.0

Values are means ± S.E.M. of data from the same eight individuals used for respirometry.

(Casey et al., 1992). Myofibrils occupy approximately 54 % and mitochondria occupy 43 % of fiber volume in honeybee flight muscles (Fig. 1; Table 2). Adenine nucleotide transport, electron transport, proton pumping and oxidative phosphorylation are made possible by enzymes and transport proteins either bound to, or associated with, the inner mitochondrial membranes or cristae. Cristae surface densities are approximately 48 m^2 cm^{-3} of mitochondrial volume (Fig. 2; Table 2). In comparison, hummingbird flight muscles possess lower mitochondrial volume densities (approximately 35 %) but higher cristae surface densities (approximately 58 m^2 cm^{-3}) (Suarez et al., 1991). These morphometric data allow the estimation of mitochondrial respiration rates during flight (Table 2). The rates per unit mitochondrial volume fall within the range reported by Casey et al. (1992) for a number of species of euglossine bee and are two- to threefold greater than the range reported for hummingbirds and four- to sixfold greater than the range reported for mammals (for a review, see Suarez, 1996). The rates per unit cristae surface area are similar to those reported in euglossines (Casey et al., 1992) and two-

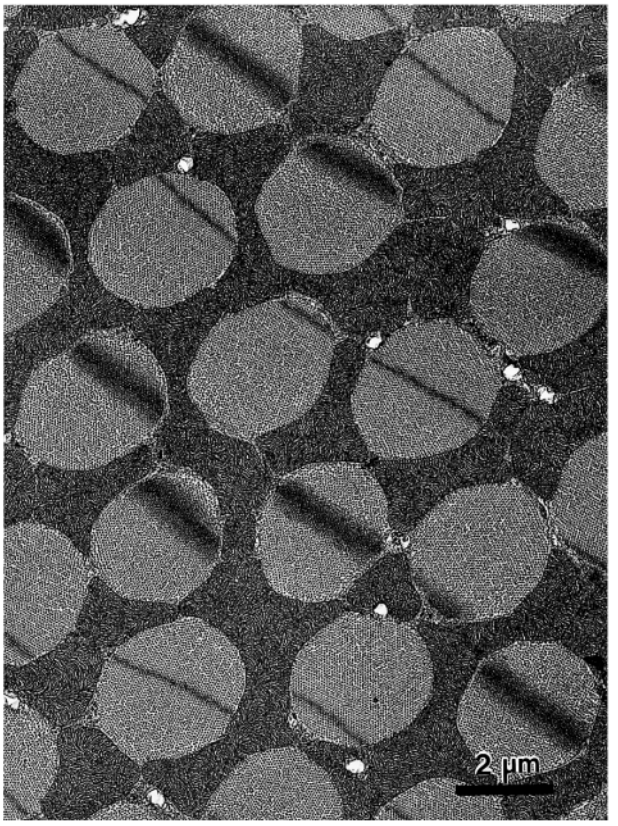

Fig. 1. Electron micrograph showing flight muscle myofibrils in cross section surrounded by abundant mitochondria. The latter appear darker because of their high cristae surface density. Small white areas are tracheae in cross section. Scale bar, 2 µm.

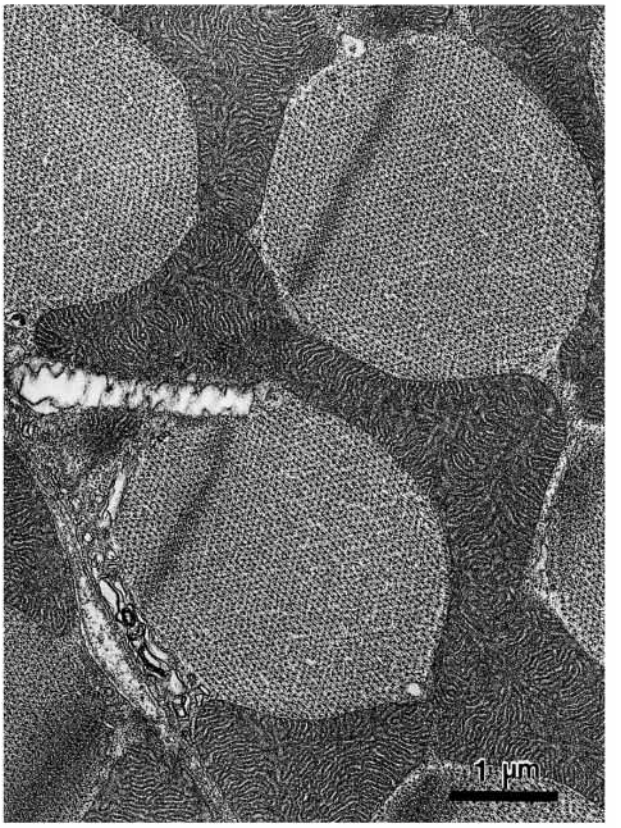

Fig. 2. Higher-magnification electron micrograph showing myofilaments within myofibrils and abundant cristae within mitochondria. Part of a tracheal tube is seen in close contact with mitochondria. Scale bar, 1 µm.

to fivefold greater than those estimated in hummingbird and mammalian locomotory muscles (for a review, see Suarez, 1996).

Respiratory chain and electron transport rates

Given the small thorax masses (<30 mg), it was not possible to perform morphometric measurements as well as spectroscopy to estimate cytochrome content (Suarez et al., 1999) on the same individuals. We therefore used recently published data concerning cytochrome content per unit mass (Suarez et al., 1999) in combination with the present data to estimate the densities of respiratory chain complexes on the inner mitochondrial membranes and their turnover (i.e. electron flux) rates during flight (Table 3). Given that 75 % of thorax mass consists of flight muscle (Nachtigall et al., 1995), the mitochondrial volume and cristae surface densities in Table 2 and assuming a uniform distribution of respiratory complexes on the inner membrane (Hackenbrock and Hammon, 1975), complexes III and IV would occur at densities of 2700 μm^{-2} and 970 μm^{-2}, respectively. Cytochrome *c*, which oxidizes complex III and reduces complex IV, is found at a density of 1466 μm^{-2}. Our estimate of the membrane density of complex III is similar to that reported by Schwerzmann et al. (1989) (3352 μm^{-2}) in cat muscle mitochondria. The cytochrome *c* content reported by Nishiki et al. (1978) and morphometric data from rat heart (Hoppeler et al., 1984) can be used to estimate a membrane density for this enzyme of 2678 μm^{-2}, a value 1.8 times greater than ours.

It is not known whether the stoichiometric relationships between respiratory enzyme complexes found in rat liver (Hatefi and Galante, 1978; Capaldi, 1982) apply to insect flight muscle mitochondria. Because it was not possible to obtain spectroscopic data for complexes I and II, we elected not to estimate the densities of these using assumed stoichiometric relationships between these and complex IV. [This is how Schwerzmann et al. (1989) obtained their values for these complexes in cat muscle mitochondria.] We confine further analysis to data derived only from our own empirical measurements.

A major reason for caution is that, while our estimate of the density of complex III on the inner membrane is similar to that of Schwerzmann et al. (1989) for cat skeletal muscle mitochondria, our value for complex IV (cytochrome aa_3 or cytochrome *c* oxidase) is an order of magnitude lower than theirs. It is therefore not justifiable to estimate the densities of complexes I and II on the bases of assumed stoichiometric relationships between these and complex IV. Nevertheless, a number of lines of evidence lead us to propose that our estimates of the content of complex IV per unit mass and of its density per unit cristae surface area are robust.

Table 3. *Densities of respiratory chain complexes and turnover rates during flight*

Respiratory chain enzyme	Number per μm^2 cristae surface area	Turnover rate (electrons complex^{-1} s^{-1})
Complex III	2699	270
Cytochrome *c*	1466	496
Complex IV	974	747

If the V_{max} value for cytochrome *c* oxidase in honeybee flight muscle homogenates, 307.7 $\mu mol\ O_2\ g^{-1}\ s^{-1}$ (Suarez et al., 1996), equivalent to 20 513 nmol electrons $g^{-1}\ s^{-1}$ (Nicholls and Ferguson, 1992), is divided by the cytochrome aa_3 content of 21.25 nmol g^{-1} (Suarez et al., 1999), we obtain a catalytic capacity (k_{cat}) for cytochrome *c* oxidase of 965 electrons s^{-1}. Given the great variability in k_{cat} estimates for this enzyme that can result from experimental error, as well as differences in assay protocol and conditions among and within laboratories (Smith et al., 1979), we do not consider our value to differ significantly from that reported from rat liver mitochondria (750 electrons s^{-1}; Gupte et al., 1984). That our estimate of the k_{cat} for honeybee cytochrome *c* oxidase falls within the expected range suggests both (i) that the estimate of enzyme content required for the calculation is accurate and (ii) that the honeybee enzyme is not a 'better catalyst' than the homologous enzyme in mammals. It is appropriate to note that Weiss et al. (1972) estimated maximal complex IV turnover rates of 67–133 electrons s^{-1} using isolated cytochrome *c* oxidase and 167–333 electrons s^{-1} using membranes from locust (*Locusta migratoria*) mitochondria. These values, however, were obtained by spectroscopic measurement of cytochrome *c* oxidation, a method known to yield lower maximal rates compared with polarographic measurements (Cooper, 1990). Hackenbrock and Hammon (1975) estimate that there are 17 000 cytochrome *c* oxidase molecules per liver mitochondrion. Given 31.4 μm^2 of inner membrane per mitochondrion (Hackenbrock and Hammon, 1975), the density of monomeric complexes would be 541 μm^{-2}. This is similar to, but lower than, our estimate of 974 μm^{-2}. We cannot comment on whether the difference is real, given the experimental errors inherent in these measurements, as well as methodological differences between laboratories. Our estimates of cytochrome aa_3 content per unit mass in honeybee thoraces (Suarez et al., 1999) are similar to those in rat ventricle (Nishiki et al., 1978), a muscle with similarly high mitochondrial volume and cristae surface densities (Hoppeler et al., 1984). Given the cytochrome aa_3 content per unit mass in rat ventricle (Nishiki et al., 1978) and the morphometric data for this tissue in Hoppeler et al. (1984), one can calculate that there are approximately 754 cytochrome *c* oxidase molecules per μm^2. Again, given experimental error and methodological differences, this value is remarkably similar to our estimate. Finally, given the turnover rate of complex IV in flying honeybees (Table 3) and density per unit cristae surface area, and given cristae surface and mitochondrial volume densities, it is possible to perform calculations to 'reconstruct' the metabolic rates presented in Table 1 to within ±10 %. Thus, we rule out the possibility that we may have significantly underestimated the content of cytochrome aa_3 (Suarez et al., 1999) or that we may have miscalculated its density on the inner membrane.

Functional implications

Honeybee cytochrome *c* oxidase displays k_{cat} values that are

unremarkable in comparison with the homologous enzyme from mammals. This result is consistent with those obtained with purified honeybee phosphofructokinase (Wegener et al., 1986) and phosphoglucoisomerase (Staples and Suarez, 1997), locust hexokinase (Storey, 1980) and moth citrate synthase (Srere et al., 1963), none of which displays a higher catalytic efficiency than the homologous enzymes from mammals. The notion that flying insects might achieve their high metabolic rates by having inherently 'faster' enzymes can therefore be laid to rest.

The respiratory chain enzymes are thought to function on the inner membrane like floating ships, randomly colliding in a sea of phospholipid (Hochli and Hackenbrock, 1979; Schneider et al., 1980). In rat liver mitochondria, lateral diffusion occurs rapidly, and collisions occur at rates in excess of the highest measured rates of electron transfer (Gupte et al., 1984). Thus, electron transfer events are 'diffusion-coupled' and not 'diffusion-controlled' or 'diffusion-limited', and ordered chains, assemblies or aggregates of respiratory chain enzymes need not be invoked to explain electron transport (Gupte et al., 1984).

Cytochrome *c* oxidase is a unique enzyme, partly because it catalyzes the reaction that leads to most of the O_2 uptake of animals during high-intensity aerobic exercise. From that data of Rothe and Nachtigall (1989), it can be estimated that, in flying honeybees, more than 90 % of the rate of O_2 uptake is accounted for by the flight muscles. This makes possible very precise estimates of metabolic flux and enzyme catalysis *in vivo*. Each cytochrome *c* oxidase molecule turns over at a rate of 747 electrons s^{-1} in flying honeybees (Table 3). This turnover rate, divided by the k_{cat} value of 965 electrons s^{-1}, yields a fractional velocity (v/V_{max}) for the enzyme of 0.774. It is likely that increased wing loading resulting from loads of nectar and pollen will further increase $\dot{V}_{O_2}$ values (Wolf et al., 1989). Flight in hypo-dense helium/oxygen mixtures has also been shown to elicit higher $\dot{V}_{O_2}$ values in euglossine bees (Dudley, 1995). Thus, cytochrome *c* oxidase may approach its V_{max} more closely when honeybees fly under conditions that elicit $\dot{V}_{O_2max}$.

The operation of cytochrome *c* oxidase at high fractional velocity implies that its substrates, reduced cytochrome *c* and O_2, are available at concentrations sufficient to cause similarly high fractional saturation of the relevant binding sites. The turnover rate of 747 electrons s^{-1} during flight establishes a minimum collision frequency (Gupte et al., 1984) between cytochrome *c* oxidase and reduced cytochrome *c*, assuming 100 % transfer efficiency. In rat liver mitochondria, the theoretical maximum collision frequency for complex IV (275 electrons s^{-1} per redox partner) exceeds the maximum rate measured by 4.6-fold (i.e. there is one transfer event for every 4.6 collisions; Gupte et al., 1984). The higher frequency of productive collisions between complex IV and reduced cytochrome *c* in honeybee flight muscles may indicate higher rates of lateral diffusion of these enzymes on the inner mitochondrial membrane.

O_2, the other substrate of cytochrome *c* oxidase and the terminal electron acceptor of the respiratory chain, must also be available to the enzyme active site at near-saturating concentrations in honeybee flight muscles. There is much disagreement among those who study mammalian muscle energy metabolism concerning the intracellular P_{O_2} in muscle fibers during exercise at $\dot{V}_{O_2max}$ (e.g. Duhaylongsod et al., 1993; Richardson et al., 1995; Honig et al., 1997; Mole et al., 1999). Unlike vertebrates, which make use of lungs and a closed circulatory system for O_2 uptake and delivery, insects possess spiracles and a tracheal system. The tracheal branches infiltrate the flight muscles and lie proximal to mitochondria (Figs 1, 2), significantly reducing diffusion distances for O_2. These features are thought to result in higher capacities for O_2 flux (Weis-Fogh, 1964) and may provide higher intracellular P_{O_2} values at the cytochrome *c* oxidase O_2-binding site. Consistent with this interpretation, we found in an earlier study that hyperoxic air does not elicit higher $\dot{V}_{O_2}$ values in flying honeybees (Joos et al., 1996).

Design of highly aerobic muscles

The similarities in respiratory enzyme packing densities on the mitochondrial inner membrane between honeybee and mammalian muscle and liver support the idea that a unit area of cristae constitutes an invariant structural unit (Taylor, 1987; Taylor et al., 1989). On the surface, this result might seem counterintuitive, given the high $\dot{V}_{O_2}$ values per unit cristae surface area in honeybees during flight. However, the high respiratory chain enzyme content per unit mass (Suarez et al., 1999) is accommodated by abundant mitochondrial volume and cristae surface densities, such that enzyme packing densities are similar to those found in mammalian heart and liver mitochondria. Honeybees achieve their high metabolic rates during flight by having a high enzyme content per unit mass and by the operation of glycolytic enzymes catalyzing non-equilibrium reactions at high fractional velocities (Suarez et al., 1996). These biochemical solutions to the problem of having to sustain high rates of metabolism also apply to cytochrome *c* oxidase and the other respiratory chain enzymes (Suarez et al., 1999). The elucidation of the mechanisms that lead to these high flux rates and the operation of these enzymes at high fractional velocities will no doubt require the application of metabolic control analysis (Fell, 1997).

There is considerable evidence suggesting that further increases in the aerobic capacities of locomotory and cardiac muscles may be limited by spatial constraints: more mitochondria means fewer myofibrils within a given fiber volume; more cristae means less matrix space within a given mitochondrial volume (see Suarez, 1996, 1998). It is tempting to speculate that more respiratory enzyme complexes per unit cristae surface area will similarly lead to diminishing returns. Schneider et al. (1980), for example, point out the need for adequate 'lateral space on the inner membrane in which the diffusion of integral proteins may occur'. Presumably, high packing densities may result in molecular traffic-jams on the inner membrane. It may be that the content of respiratory chain enzymes per unit cristae surface area represents another

example of 'enough but not too much' (Diamond, 1991) in biological design.

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