ABSTRACT

During hovering flight, insects reach rates of energy production that are among the highest measured in the animal kingdom, making them ideal organisms in which to study the evolution of energy metabolism. The work presented in this thesis utilised a lineage of orchid bees that covers a 20-fold range in body mass (approximately 50 - 1000 mg) to address this issue. Body mass strongly affects flight energetics and contributes to variation in metabolic rate between species, providing a useful model to investigate the correlated evolution of whole-animal flight energetics and the design of metabolic pathways involved in energy production.

At the whole-animal level, I showed that the mass-specific metabolic rate ranged from 114.4 to 37.4 ml CO$_2$ hr$^{-1}$ g$^{-1}$ in small and large species respectively. Similar variation in hovering flight wingbeat frequency was found for small and large species, ranging from 250 to 86 Hz, suggesting a direct relationship between these two variables. Wingbeat frequency variation was further shown to be explained by wing size and wing loading. The method of phylogenetically independent contrasts was used to demonstrate the correlated evolution of body mass independent wing loading, wingbeat frequency and mass-specific metabolic rate. The biochemical correlates associated with metabolic rate variation were also studied in orchid bee flight muscles. The activity of several enzymes of glycolysis, Krebs cycle and the electron transport chain was measured. Only for the enzyme hexokinase (HK) was there a direct relationship between its activity and the mass-specific metabolic rate during hovering flight. The activity of HK ranged from 73.8 to 19.4 U g$^{-1}$ thorax in small and large species respectively. The activity of HK was correlated with mass-specific metabolic rate independent of body mass or phylogenetic relatedness. The role of HK as a regulator of glycolysis and its high control of overall pathway flux appears to explain this relationship. These studies represent the first example of the biochemical alterations needed (over evolutionary time) to support changes in animal size, which is explained by the form and function associated with
flight. A model addressing the scaling of metabolic rate with animal size, including the concept of multiple contributors to metabolic rate, was developed. I proposed the concept of an allometric cascade, which integrates various effects of size on the multiple contributors to metabolism involved at various levels of organisation, and tested it using data from mammals. Together, the work presented in this thesis demonstrates that the effect of body mass on animal physiology has many facets, reflecting the various aspects of animal form and function.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AM ATPase</td>
<td>Actomyosin ATPase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>$b_i$</td>
<td>Scaling exponent of physiological contributors to metabolic rate</td>
</tr>
<tr>
<td>BMR</td>
<td>Basal metabolic rate</td>
</tr>
<tr>
<td>$c_i$</td>
<td>Control coefficient</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>cyt $b$</td>
<td>Cytochrome b gene</td>
</tr>
<tr>
<td>$D_{\text{lung}}$</td>
<td>Pulmonary diffusion</td>
</tr>
<tr>
<td>$D_{\text{tissue}}$</td>
<td>Capillary-mitochondria diffusion</td>
</tr>
<tr>
<td>[E]</td>
<td>Enzyme concentration</td>
</tr>
<tr>
<td>GP</td>
<td>Glycogen phosphorylase</td>
</tr>
<tr>
<td>GPDH</td>
<td>Glycerol 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>MMR</td>
<td>Maximum metabolic rate</td>
</tr>
<tr>
<td>$M_b$</td>
<td>Body mass</td>
</tr>
<tr>
<td>$M_{\text{mito}}$</td>
<td>Cytosolic and mitochondrial metabolism</td>
</tr>
<tr>
<td>MR</td>
<td>Metabolic rate</td>
</tr>
<tr>
<td>MR$i$</td>
<td>Energy turnover rate of individual metabolic processes</td>
</tr>
<tr>
<td>$n$</td>
<td>Wingbeat frequency</td>
</tr>
<tr>
<td>$P_w$</td>
<td>Wing loading</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PGI</td>
<td>Phosphoglucoisomerase</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>PIC</td>
<td>Phylogenetically independent contrast</td>
</tr>
<tr>
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<td>Hierarchical control coefficient</td>
</tr>
<tr>
<td>$Q$</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>$R$</td>
<td>Forewing length</td>
</tr>
<tr>
<td>$S$</td>
<td>Total wing area</td>
</tr>
<tr>
<td>TR</td>
<td>Trehalase</td>
</tr>
<tr>
<td>$V_A$</td>
<td>Alveolar ventilation</td>
</tr>
<tr>
<td>$\dot{V}_{\text{co}_2}$</td>
<td>Carbon dioxide production rate</td>
</tr>
<tr>
<td>$\dot{V}_{\text{co}_2}^*$</td>
<td>Mass-specific carbon dioxide production rate</td>
</tr>
<tr>
<td>$\dot{V}_{O_2}$</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>$\dot{V}_{O_2}\text{max}$</td>
<td>Maximum aerobic capacity</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Enzyme maximum activity</td>
</tr>
<tr>
<td>$%V_{\text{max}}$</td>
<td>Enzyme fractional velocity (pathway flux / $V_{\text{max}}$)</td>
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J’aimerais dédier ces travaux à Peter Hochachka. Avant même de le connaître, Peter m’a inspiré l’étude de l’adaptation biochimique. Après avoir travaillé avec lui, il m’a non seulement transmis la passion et le plaisir que peut apporter l’exploration scientifique, mais il m’a également fait vivre des expériences de vie irremplaçables. Merci pour m’avoir démontré la haute voltige de la science, de m’avoir donné les moyens et la liberté d’explorer mes idées, et d’avoir été si généreux jusqu’à la fin. J’aimerais également remercier Raul Suarez qui a été aussi influent dans cette quête scientifique. Les nombreuses discussions échangées et les expériences partagées, comme mes visites à Santa Barbara et nos voyages de recherche au Panama, m’auront beaucoup appris. Merci à Patricia Schulte qui a joué un rôle crucial en acceptant de me prendre sous sa charge lors du décès de mon directeur, Peter Hochachka. Elle a été extrêmement généreuse et a beaucoup contribué à la fin de ces travaux. Le support et l’amitié de ces trois personnes ont fait de ces travaux de thèse un souvenir inoubliable. J’aimerais finalement remercier Pierre Blier, qui m’a fait découvrir ce domaine de recherche et m’a aidé et poussé à devenir l’étudiant de Peter. Ce fut toute une expérience, tout un apprentissage.

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Chapter 4 of this thesis includes text modified from the following previously published articles:


CHAPTER 1
GENERAL INTRODUCTION
EVOLUTIONARY PHYSIOLOGY AND INSECT FLIGHT ENERGETICS

Energy metabolism, locomotion and body size

Animal locomotory performance depends on the design of physical structures (fins, limbs, wings), as well as the physiological and biochemical "machinery" that drives these structures. Energy requirements during locomotion will be affected by many factors such as body size, locomotory mode and ecological niche. Thus, animals vary widely in their athletic performance, which contributes to their physiological diversity. The evolution of the capacity for locomotion may place selective pressures on the physical structures associated with locomotory performance, as well as the physiological and biochemical machinery involved. The study of the evolution of energy metabolism associated with locomotion should therefore integrate various aspects of an animal's physiology within an evolutionary context.

One aspect of animal species diversity that greatly influences locomotion is body size. The effect of body mass on animal morphology, biomechanics and physiology related to locomotion has been a fruitful area of integrative research. For example, if geometric proportions of mammals are conserved, the length of limbs (l) should be proportional to one-third power of its mass, which appears to be the case for mammal limb bones (Alexander, 1979). From this relationship, a body mass power function can be predicted for stride frequency ($M_b^{-0.33}$), muscle mass ($M_b^{1.0}$), and work per kg per km ($M_b^{-0.33}$) (Schmidt-Nielsen, 1984). Empirical measures showed that the cost of running scales close to the -1/3 power in mammals, reptiles and birds (Schmidt-Nielsen, 1984). Studies have further examined body mass effects on physiological correlates associated with locomotion. A series of studies by Taylor, Weibel and colleagues (Weibel, 2000), investigated maximal aerobic exercise in mammals ranging from shrews to horses running on treadmills. Such studies, however, analyse the relationship between
continuous variables, using species mean values as data points. Development of the comparative method by evolutionary biologists (Felsenstein, 1985; Harvey and Pagel, 1991; Garland et al. 1992), has shown the importance of incorporating phylogenetic information in species data comparisons aiming to study adaptation and allometry (Harvey and Pagel, 1991; Nunn and Barton, 2001).

*Evolutionary physiology and allometry*

One goal of comparative physiology and biochemistry, described early on by Prosser (1950), is to elucidate evolutionary relationships of animals by comparing physiological and biochemical characteristics. The traditional approach is to compare species that represent different (usually opposite) life-styles and infer if the observed mechanism is adaptive. This method of inferring an evolutionary explanation for observed differences follows “the adaptationist programme”, in which no rigorous analytical tools are used (Gould and Lewotin, 1979; Garland and Carter, 1994). Garland and Adolph (1994), pointed out that phenotypes are likely to vary between species solely due to random mutation and genetic drift. Thus, there would be 50% probability that physiological measurements made on two species will differ in the expected direction, even if this difference is not due to natural selection (Garland and Adolph, 1994)! This analysis points to the need for more rigorous experimental and analytical methods for the investigation of evolution at the physiological level, and this need has led to the development of evolutionary physiology, as a field unto itself (Diamond, 1992, 1993; Garland and Carter, 1994). Mangum and Hochachka (1998) suggested that one preferred goal of the discipline of comparative physiology and biochemistry would be to blend mechanistic and evolutionary physiology.
The field of evolutionary physiology generally focuses on the evolution of physiological traits using four investigative approaches: genetic analyses and manipulation, selection studies, manipulation and study of phenotypic plasticity, and phylogenetically based comparisons (Feder et al., 2000). Genetic analyses and manipulation can be summarised as the physiology-to-genetics and gene-to-physiology approaches, the former approach applying techniques of quantitative genetics to estimate the heritability of different functional characters (Garenc et al., 1998, Garland et al., 1990, Dohm et al., 1996, Tsuji et al., 1989, Sorci et al., 1995), and the latter examining the performance and fitness consequence of a single or selected genes (Powers and Schulte, 1998, DiMichele et al., 1991; Kohen et al., 1980, 1983; Watt et al., 1983; Barnes and Laurie-Ahlberg, 1986). Selection studies use reproductive success or differential survival, in natural environments or the laboratory, to investigate the consequence of a selection process on physiological characters (Miles, 1987; Jayne and Bennett, 1990; Bennett and Huey, 1990; Bennett et al., 1992; Bennett and Lenski, 1993, 1996; Leroi et al., 1994a; Gibbs et al., 1997; Bennett and Lenski, 1999). Other approaches manipulate phenotype (Sinervo and Huey, 1990; Buchmiller et al., 1993; Hammond et al., 1996a,b) or investigate phenotypic plasticity (Leroi et al., 1994b; Huey and Berringan, 1996) of physiological characters. Finally, recent studies have employed phylogenetically based comparisons to look at various aspects of physiological adaptation (Block et al., 1993; Losos, 1990; Garland, 1994; Mottishaw et al., 1999; Walton, 1993; Pierce and Crawford, 1997).

An analytical method used to compare species and test for correlated evolution of traits has been developed by Felsenstein (1985), and applied to evolutionary physiology by Garland et al. (1992). This method consists of incorporating phylogenetic information into the statistical analyses of correlated evolution of continuous traits (physiological traits in our case). Without using such analytical method in comparative studies, species data points are not independent,
both biologically and statistically. Using species averages in conventional statistical analysis of continuous traits assumes that the data points are independent and that the variance is equal. To fulfil the first assumption, the relationship among the species used assumes a star phylogeny, with all species evolving from a common ancestor (Fig. 1.1A). Instead, species are related in a hierarchical manner, where species are organised in lineages that are more likely to share similar characters (Fig. 1.1B). Such group-specific differences in character value can be explained by various evolutionary mechanisms other than adaptation, such as genetic drift or phylogenetic inertia. The variation in character values associated with phylogeny is termed phylogenetic signal, which can be detected using the method of phylogenetically independent contrasts.

To correct for nonindependence of taxa, this kind of analysis requires (1) data for 2 or more phenotypic traits for a series of species, (2) the cladistic relationships of these species, and (3) phylogenetic branch length in units of expected variance of change (Garland et al., 1992). A contrast is defined as the difference in a phenotype X between pairs of adjacent tips (e.g. \( X_1 - X_2, X_3 - X_4, \) etc), or nodes (e.g. \( X_7 - X_8, X_3 - X_6, \) etc). Such contrasts take into account variations among members of a same branch, which can then be analysed as \( n - 1 \) independent events. Felsenstein’s analysis also corrects for the nonequal variance that may occur due to differential rates of evolution of each character. It assumes that a character evolves in the same way that the movement of a molecule is randomly affected by thermal noise (i.e. Brownian motion); the displacement after a period of time \( v \) is the sum of small displacement that can be either positive or negative. In a phylogenetic tree, the variance \( (s^2_X) \) of each character is proportional to \( v \), the branch length, so that the variance of a character \( X \) after \( v \) time is equal to \( s_x^2 v \), and for a character \( Y \) is equal to \( s_y^2 v \). By dividing a contrast by its standard deviation, it is possible to standardise in unit variance, which is comparable among characters measured; it is
the standardised contrast. This standardised contrast is the value corrected for nonindependence and nonequal variance due to phylogeny. These data can then be properly treated statistically, to detect correlation between characters that may have evolved in parallel, to ultimately detect adaptation.

*Orchid bees as a model*

In this thesis, I used a lineage of hovering insects to study the evolution of biochemical correlates of flight energetics. Insects that perform hovering flight require a great amount of energy to meet the power output necessary for lift. In fact, the oxygen consumption rate of hovering insects is among the highest measured in animals (Sacktor, 1976). Therefore, the flight muscles of hovering insects must possess a series of physiological adjustments that enable them to maximise rates of aerobic energy production. Thus, these animals represent an excellent model to study the evolution of energy metabolism. One group of hovering insects, the orchid bees (Apidae; Euglossini), contains many closely related species that can be found in the same environment (tropical forest), which allows one to perform phylogenetically based comparisons, while controlling for environmental factors such as temperature. In addition, orchid bee species vary widely in body mass, which has been shown to greatly affect their flight energetics (Casey et al., 1985). This system thus provides a great opportunity to investigate the diversity of locomotory performance and its physiological and biochemical bases.

The Euglossini is the only tribe of solitary bees from the sub-family of the Apinae (family Apidae), which also includes the tribes Bombini (bumblebees), Apini (honeybees), and Melopini (stingless bees). Roubik and Hanson (2004) and Cameron (2004) reviewed the biology and phylogeny of this group of corbiculate bees, which includes approximately 190 species grouped in 5 genera. The genus *Aglae* is rare and includes only 1 species. Among the
other 4 genera, 2 are pubescent and 2 are glabrous with metallic colors. Bee species of each
genus also share similar characters such as body mass. For example, among the 190 species,
104 are in the genus *Euglossa*, which includes species body mass ranging from approximately
50-200 mg, while the genus *Eulaema* includes 15 species covering approximately 400-1200 mg
in body mass. Phylogeny thus clearly influences the grouping of characters such as body mass
and associated characters, which stresses the importance of accounting for phylogenetic signal
when making comparisons of physiological traits. Unfortunately, relationships among species
of this group are not yet well resolved, thus one objective of this work is to develop a
hypothesis regarding the phylogenetic relationships of the species under investigation.

*Background on insect physiology*

**Flight muscles.** In flying insects, two principal types of flight muscle have evolved:
synchronous (used by orders such as Lepidoptera, Orthoptera), and asynchronous (used by
Hymenoptera, Diptera, Coleoptera, etc). As the names imply, the muscle contraction frequency
of the former is synchronised with the nerve impulse frequency, while in the latter the firing of
each neural signal results in several contractions. Synchronous muscles follow the “typical”
muscle activation-contraction coupling, where the sarcoplasmic reticulum (SR) releases and
then sequesters Ca$^{2+}$ before the next contraction. Sarcoplasmic reticulum volume in various
insects is inversely proportional to twitch duration (Josephson, 1975), and a larger volume of
SR increases the frequency of muscle contraction, up to approximately 100 Hz. However, as SR
volume increases, space available for myofibrils decreases, reducing the capacity for force
generation. Higher wingbeat frequencies have become possible due to a muscle activation
mechanism that bypasses the SR limitation. Asynchronous muscles achieve this because the
nervous system provides the initial stimuli, but does not maintain the rhythmic muscle
contraction. In fact, in an asynchronous muscle, the ratio of action potential: muscle
contractions varies from 1:5 to 1:40 (Chapman, 1998). Stretch activation mechanisms maintain the contraction frequency, and enable these muscles to reach high contraction frequencies (up to 1000 Hz), without sacrificing force generation (volume occupied by myofibrils). The evolution of asynchronous muscle thus represents a major physiological innovation to achieve hovering flight, since high muscle contraction frequencies are possible in conjunction with sufficient force generation and a high rate of energy production.

*Tracheal system.* A feature of terrestrial insects that enables them to reach such a high rate of energy production is the use of a tracheal system for oxygen delivery. This system consists of an air convection and diffusion step to transport gases within the tracheal system, and a diffusion step from the tracheole to the mitochondria. The unique characteristic of this system is that delivery of $O_2$ from the environment to the tracheal ending takes place in an aerial medium. Oxygen diffuses 100 000 times faster in air than in water or tissue (at normal temperature and pressure) (Weis-Fogh, 1964a). Hence, even if the path of $O_2$ in the tracheal system is much longer than the tissue diffusion distance, diffusion of $O_2$ from the tracheal ending to the mitochondria is more likely to be limiting than diffusion from the spiracle (trachea opening) to the tracheal ending (Chapman, 1998). In primitive insects like Odonates (dragonflies), the tracheoles are restricted to the outside of the muscle fibre, which limits the actual size of the fibre close to a theoretical maximum diameter of 20μm (Weis-Fogh, 1964a). Most flying insects, such as Hymenoptera, have their tracheoles functionally internal (deeply indented in the muscle), which allows the muscle fibres to be much bigger.

In most metabolically active insects, the delivery of $O_2$ in the tracheal system is not only based on diffusion, but also on convection. Insects ventilate the trachea, mainly by changing the volume of the system by using flexible air sacs. The insect tracheal system is not permanently opened to the external environment, as they can ventilate in episodes by opening and closing
the spiracles, mainly for water conservation. Depending on the intensity and the nature of the activity, different ventilation patterns are observed in insects. In Hymenoptera and Diptera during flight, an increase in abdominal pumping is performed to enable a good O\textsubscript{2} supply to the thoracic flight muscle (Weis-Fogh, 1964b). Nevertheless, no limitation of oxygen delivery by the tracheal system has been shown so far in insects such as honeybees (Harrison et al., 2001; Joos et al., 1997) and grasshoppers (Greenlee and Harrison, 2004).

**Flight mechanics and energetics.** The cost of flying is extremely high for insects, especially hovering insects. The total power required for flight is the sum of aerodynamic power plus the inertial power. The aerodynamic power can be defined as the induced power needed to accelerate the air downward to balance the animal's weight, and the profile power to overcome the drag of the wings. Inertial power is the cost of accelerating the wing (Ellington, 1984) and depends on the elastic storage of the flight apparatus. Therefore, the total power required for flight depends on many factors such as wing size and shape. Understanding determinants of power output will provide the framework to understand metabolic power diversity and evolution.

Wingbeat frequency is a major component of power output. Pennycuick and Rezende (1984) established that the power output of a muscle of similar mass-density is proportional to the cycle frequency, and the force generation properties of the muscle, which depends on the internal myofibrillar stress and strain of the muscle. The variation in wingbeat frequency has been studied in orchid bees by Casey and colleagues (1985, 1992). The wingbeat frequency varied 3-fold in 9 species of orchid bees, declining with an increase body mass (Casey et al., 1985). The hovering flight mass-specific metabolic rate measured in the same species showed a similar scaling pattern, suggesting the strong relationship between cycle frequency and metabolic power. In addition, Casey et al. (1985) showed that wingbeat frequency appeared to
be related to wing morphology, which was suggested to differ between genera. The correlations suggested by Casey et al. (1985), however, did not incorporate the effect of body mass covariation and phylogenetic relatedness. Orchid bees can thus be useful to investigate the evolutionary correlates of wingbeat frequency variation.

Muscle ultrastructure. To enable insect flight muscle to produce the necessary amount of energy for flight, this muscle ultrastructure is proportioned differently relative to the muscles of vertebrates. Both synchronous and asynchronous muscles contain extremely high mitochondrial volume. The remaining volume is mainly occupied by myofibrils and SR. In Euglossine bees, it has been reported that the mitochondrial fraction is inversely proportional to body mass, and the myofibrillar proportion increases with size (Casey et al. 1992). The mitochondrial content was found to vary from 30% for a 1 g bee to 43% for a 100 mg bee. The allometry of mitochondrial volume follows the allometry of mass-specific aerobic metabolism (negative scaling of mass-specific parameters), as expected. One might think that the variation in O₂ consumption is proportional to the variation in mitochondrial volume. In other words, the mitochondria are the same, and to vary the O₂ uptake of a muscle, the muscle mitochondrial content (size or number) is modified. While it appears that mitochondrial content is modified (Casey et al. 1992), it does not account for the entire variation in O₂ consumption rate. Casey et al. (1992), calculated that the mitochondrial respiration rate is 16 ml O₂ (cm³ min)⁻¹ for a 0.1g bee and 6.2 ml O₂ (cm³ min)⁻¹ for a 1 g bee. The cristae surface density does not seem to be the cause of this variation, since it was shown by these authors to be independent of body size (50 m² cm⁻³). It thus seems that the variation in the quantity of structure does not fully account for the differences observed.

Biochemical level. During insect flight, more than 90% of the O₂ consumption of the whole animal is due to flight muscle (Rothe and Nachtigall, 1989). The flight muscle is composed of only one fibre type, which simplifies its study. Additionally, these muscles
exclusively use hexoses as fuels during flight, as demonstrated by the measured respiratory quotient (RQ = \( \frac{V_{co_2}}{V_{o_2}} \)) of 1 (Rothe and Nachtigall, 1989). These muscles have no detectable \( \beta \)-hydroxyacyl CoA dehydrogenase activity (a fatty acid catabolic enzyme), and homogenate preparations cannot catabolise fatty acid substrates (C.-A. Darveau, D. O’Brien and R.K. Suarez, unpublished data). Furthermore, glycogenolytic capacity is too low to support flight (Crabtree and Newsholme, 1972), so the hexose source comes directly from dietary sucrose (Gmeinbauer and Crailsheim, 1993). Thus, insect flight muscle presents a simplified model in which to study energy metabolism.

The energy metabolism of the Honeybee (*Apis mellifera*) has been recently investigated through a series of studies by Suarez and colleagues (1996, 1998, 1999, 2000). As mentioned previously, hovering insect muscle possesses high mitochondrial content with densely packed cristae. Still, the \( O_2 \) consumption per unit cristae surface area is higher than any other animal measured. Suarez et al. (2000), tested whether the higher rate of \( O_2 \) consumption per cristae is due to higher packing of enzymes per unit cristae surface area, or if the enzymes work at higher fractional velocity (\( \%V_{max} \)). They concluded that the enzyme packing densities are similar among the honeybee muscle compare with mammalian muscle and liver, supporting the idea that a unit of cristae surface is an invariant structure (Taylor, 1987). They estimated the fractional velocity of cytochrome c oxidase, and observed that this enzyme works closer to Vmax than in any other muscle known (Suarez et al., 1996, 1999, 2000). It appears that in honeybees, many other enzymes also work close to their Vmax (Suarez et al., 1997), such as certain glycolytic and oxidative phosphorylation enzymes (Suarez et al., 1996, 1999). In addition, Suarez et al., (1997) showed that a gradient of \( \%V_{max} \) of several enzymes (glycogen phosphorylase, hexokinase, phosphofructokinase) appeared to be correlated with glycolytic flux rate in animals ranging from fish to honeybee. This range in \( \%V_{max} \) among distantly related
groups may indicate an adjustment of fractional velocity at which enzymes work with an increase in flux rate, but the adaptive value of this mechanism can only be assessed by looking at a smaller evolutionary scale (genus or species level).

**Thesis objectives**

Many levels of physiological organization can potentially be involved in the adjustments required with variation in locomotor performance, and the nature of these adjustments is likely to vary depending on the evolutionary time scale. For example, in insect flight muscle over broad evolutionary time scales we observe adjustments such as the major transition from synchronous to asynchronous flight muscle. At shorter evolutionary time scales, much of the variation in flight performance within lineages of flying insects may involve quantitative adjustments of the physiological determinants of flight energetics, rather than fundamental alterations in structure or function. The 20-fold range of body mass covered by the orchid bee lineage imposes distinct effects on flight energetics and biomechanics (Casey et al., 1985), providing an excellent model to investigate the physiological adjustments with changes in hovering flight energetics.

The main objective of my research was to investigate the evolution of energy metabolism. The first part of this thesis aimed to link form and function associated with hovering flight. The relationship between body mass, wing morphology, wing kinematics and metabolic rate during hovering flight was suggested to be correlated and possibly coevolved. By incorporating phylogenetic information derived from cytochrome b gene sequence, the correlated evolution between body mass, wing length and area, wing loading, wingbeat frequency and mass-specific metabolic rate was tested. For these measurements performed at the whole-animal level, I tested the hypothesis that the wingbeat frequency directly determines
metabolic rate during hovering flight, and that this relationship is independent of phylogenetic history. In addition, I hypothesised that wing morphology is related and explains wingbeat frequency variation. The second part of this research investigated the correlated evolution between metabolic rate and metabolic pathways, by measuring the activity of enzymes involved in glycolysis, the Krebs cycle and the electron transport chain, and comparing the scaling of these parameters to that of metabolic rate. At present there is only a limited conceptual framework with which to make predictions regarding which component of the biochemical pathways is most likely to be involved in setting metabolic rate, and thus be involved in its evolution. As a result, I chose to assess the activities of the enzymes across several metabolic pathways, to test the hypothesis that all of the enzyme activities measured will scale in parallel to mass-specific metabolic rate. Finally, I developed the concept of an allometric cascade as a model for explaining the functional basis of the scaling of metabolic rate with body size in animals.
Figure 1.1. Hypothetical relationship between two continuous variables, body mass and a physiological variable. (A) Species evolving from a common ancestor with a star phylogeny are independent data points, where X and Y are correlated, independent of phylogenetic relationships. (B) In this hierarchical organisation of species, data points are grouped in 2 species clusters a and b, where X and Y variables are no longer correlated when corrected for phylogenetic grouping.
CHAPTER 2
EVOLUTION OF HOVERING FLIGHT ENERGETICS IN A LINEAGE OF ORCHID BEES (APIEDEA: EUGLOSSINI): LINKING FORM AND FUNCTION

Introduction

Flying insects achieve some of the highest rates of aerobic energy metabolism known in the animal kingdom (Saktor, 1976; Suarez, 2000). A major evolutionary innovation that contributed to the impressive flight abilities and evolutionary success of many species was the development of asynchronous flight muscles. These permit high contraction frequencies by decoupling muscle activation and contraction systems. Even among asynchronous flyers, a wide range of flight performance is found, involving finer evolutionary adjustments in form and function associated with flight.

One group of asynchronous flyers, the orchid bees (Apidea: Euglossini), includes approximately 190 species found in the tropical Americas (Cameron, 2004; Roubik and Hanson, 2004). These vary in body mass over a 20-fold range from about 50 mg to greater than 1 g. Body size is considered to be one of the most important properties of animals that profoundly influences many (and perhaps most) aspects of their structure and function (Schmidt-Nielsen, 1984). Many investigations have addressed the factors that drive mass-related scaling relationships, but few have considered body mass effects on both form and function. In a series of pioneering studies, Casey et al. (1985) exploited the naturally-occurring size variation in orchid bees to investigate the allometric scaling of flight biomechanics and energetics. They found that mass-specific metabolic rates and wingbeat frequencies decline with increasing body mass in orchid bees. However, they found that body mass alone does not fully account for the species variation in metabolic rate or wingbeat frequency, which led them to propose that other traits associated with phylogenetic status must be partly responsible for the observed interspecific variation. The orchid bees, therefore, represent an excellent model
with which to further examine the linkages among morphology, kinematics and energetics in
the context of phylogenetic relationships (Felsenstein, 1985; Harvey and Pagel, 1991; Garland
et al., 1992). In this chapter, I extend the analysis of Casey et al. (1985) to a larger number of
species and looked at the relationship of metabolic rate to wing morphology, kinematics and
flight muscle energetics. In addition, I incorporated a phylogenetic perspective by using the
method of independent contrasts (Garland et al., 1992). To incorporate phylogenetic
information into the analysis of continuous traits requires a hypothetical tree topology and
associated branch lengths (Garland et al., 1992). In this study, I developed a phylogenetic
hypothesis to account for the relationships among orchid bees using partial sequence of the
cytochrome b gene (cyt b), and used it to analyse metabolic rate evolution. Using
phylogenetically independent contrasts, I analysed the correlated evolution of body mass, wing
morphology, wingbeat frequency, and the energetic cost of hovering flight. This analysis
provides a functional framework to understand the relationship between species size and
metabolism.

Materials and Methods

Study site and orchid bee collection

The 32 species used to generate the molecular phylogeny were collected at various sites
in Panama by David W. Roubik and were either preserved in ethanol or frozen (-80°C). Data on
flight energetics were obtained from 86 individuals of 18 species, representing a subset of the
32 species included in the molecular phylogenetic work. The metabolic rate and wingbeat
frequency measurements were carried out in the field during the transition from dry to wet
season in June 2003, at a site made available by the Smithsonian Tropical Research Institute
(Panama). This was located near the centre of the 15 km² island of Barro Colorado, in the
Panama Canal. Ambient temperature when measurements were performed ranged from 25 to 30°C. Male orchid bees were attracted using the chemical lures cineole and methyl salicylate (Sigma Chemicals) (Dressler, 1982) applied to blotting paper. These are naturally occurring compounds found in the fragrances collected by male Orchid bees from various Orchid species, as well as other floral and non-floral sources (Roubik and Ackermann, 1987). Individuals were collected using insect nets, collection vials, or by hand when resting on the paper containing the chemical lures.

_Molecular phylogeny_

To assess the phylogenetic relatedness of the various species used, I generated hypothetical phylogenies based on partial sequences for the mitochondrial gene cytochrome b. I extracted total DNA from individuals of 32 species, mainly from the thorax (sometimes including several legs), using the DNeasy DNA extraction kit (Qiagen), following the manufacturer's recommended homogenization procedure for insects.

The amplification procedure used was modified from Koulianos and Schmidt-Hempel, (2000). Using their cytochrome b primers, I amplified a 716bp fragment of _cyt b_. The conditions used that yielded the highest amplification success for most species was as follows: denaturation at 94°C for an initial 2 min and then 40 cycles of 1 min denaturation at 94°C, annealing at 43°C for 1 min, extension at 72°C for 1 min, followed by a 5 min final extension (Perkin-Elmer DNA Thermal Cycler); _Taq_ polymerase (Invitrogen) 2.5 U, MgCl$_2$ 1.5mM, dNTP 0.2mM, primers 0.5μM. For some species, the yield of amplification product was low. This required slight modification of time, temperature, MgCl$_2$ and Taq concentrations. DNA product was purified using Quiaquick purification columns (Qiagen). Sequencing was performed by the University of British Columbia Nucleic Acid and Protein Sequencing Unit
using an ABI 377 automated sequencer. The primers used for sequencing were the same as those used for amplification. In most cases, one individual was sufficient to provide a sequence.

The cytochrome b sequences (~ 650 bp) obtained for 32 species were aligned using ClustalX. Sequences were also obtained from GenBank for *Eulaema meriana* (Genbank accession number AF181614), *Eulaema bombiformis* (AF002728), *Eufriesea caerulescens* (AF181613), in addition to the selected outgroups: *Xylocopa virginica* (AF181618), *Apis mellifera* (NC_001566), *Bombus hyperboreus* (AF066968), *Trigona hypogea* (AF181617) and *Melipona bicolor* (NC_004529). Trees were rooted with *Xylocopa virginica* which is hypothesized to be a sister clade (Ascher and Danforth, 2001).

Aligned sequences were imported into PHYML for maximum likelihood analysis. Several sequence divergence models were executed, and a general time reversal with gamma distribution was selected based on log-likelihood score. The support for the nodes was obtained using 1000 bootstrap replicates using PHYML and SEQBOOT and CONSENS from PHYLIP 3.57c (Felsenstein, 1995). Additional analyses were performed using the neighbour-joining distance method using MEGA version 2.1 (Kumar et al. 2001), and Bayesian analysis using MrBayes 3.0 (Huelsenbeck and Ronquist, 2001).

**Respirometry and wingbeat frequency measurements**

Respirometry measurements were conducted immediately after capture to minimize time-dependent changes in flight 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 per min by a FOX flow-through field respirometry system equipped with a CO₂ analyzer (Sable Systems, Henderson, Nevada).
Because bees fuel flight exclusively with carbohydrates (Crabtree and Newsholme, 1972; Suarez, 2000), the \( \dot{V}co_2 \) values measured are equivalent to \( \dot{V}o_2 \) (i.e., RQ = 1, Roth and Nachtigall, 1989). I have verified that these generalizations, based on information from the literature, also apply to Orchid bees when measurements of both \( \dot{V}o_2 \) and \( \dot{V}co_2 \) were available (C.-A. Darveau, K. Welch and R.K. Suarez, unpublished). 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 reliable results. Data acquisition and analysis were performed using Datacan (Sable Systems).

Wingbeat frequency measurements were performed simultaneously using an optical flight detector (Qubit systems, Kingston, Ontario), mounted on a ring stand and positioned beneath the respirometry chambers. The instrument was linked to a portable computer and the signal was acquired and analysed using TRex (Moore Scientific). The wingbeat of an insect performing hovering flight in the chamber was detected by the photocell. Two seconds intervals were analyzed for fundamental frequency and data for each individual was averaged for the initial 30 sec of flight.

Wing morphology

The individuals were frozen on dry ice, stored at -80 C, and brought back to the laboratory for morphological measurements. From each individual, one pair of wings was removed and flattened between microscope slides. A digital image of the wings on a 1 cm grid background was taken for image analysis using Scion image.
Data analysis

All data are presented as species mean values, with error bars representing the standard deviation of individual measurements. However, both individual data and species means were analysed. The effect of body mass on the different characters was tested using the least squares linear regression performed on log transformed data to obtain the power equation \( Y = aM^b \). Further analyses of wingbeat frequency and mass-specific metabolic rate were performed using stepwise regressions to test the effect of body mass, forewing length, total wing area and wing loading. In addition, I corrected for body mass co-variation using residuals analysis.

Analysis of phylogenetically independent contrasts was conducted using the PDAP (Midford et al., 2003) module in Mesquite (Maddison and Maddison, 2004). Standardized independent contrasts were obtained from the log transformed character data, and presented using the maximum likelihood tree I obtained from cyt b sequence information (Fig. 2.1). This phylogenetic tree was pruned to include only the 12 species from which we succeeded in collecting a complete set of flight data (Fig. 2.2). I also performed all analyses using the hypothesised trees obtained from other phylogenetic analyses I performed (neighbour-joining, Bayesian, maximum parsimony). The results of these analyses were qualitatively the same (data not shown). To further test for phylogenetic tree topology and branch length uncertainty (see Garland et al., 1992; Garland et al., 1993; Martins and Housworth, 2002), I implemented the analysis of character data using 10,000 trees generated from a Bayesian analysis and reported correlation coefficient frequency distribution of standardized independent contrasts using Mesquite. I analysed the relationship among independent contrasts with the same series of tests I used for the conventional analysis. I first tested body mass scaling effects on the different characters, using least squares linear regression through the origin performed to analyse standardized contrasts (Garland et al., 1992). I then carried out stepwise regression through the
origin to analyse the independent contrasts of wingbeat frequency and mass-specific metabolic rate, to test for the effect of the variable body mass, forewing length, total wing area and wing loading. Finally I statistically controlled for body mass using the residuals obtained from the independent contrasts body mass regressions, and plotted the residuals and tested for regression through the origin (Garland et al., 1992). All analyses were performed first using a model of gradual evolution, where characters usually experience greater changes along longer branches (Garland et al., 1993). I ensured that branch lengths adequately standardised the contrasts by plotting the absolute value of standardised independent contrasts and their standard deviation (Garland et al., 1992). The raw branch lengths obtained from \textit{cyt b} genetic distances were used, but several branch length transformations (Grafen, Pagel, Nee, logarithmic) were also tested and yielded the same results (not shown). Speciational model of evolution, where changes occur with a speciation event, was simulated by setting all branch lengths to 1 (Garland et al., 1993).

Results

\textit{Molecular phylogeny}

I aligned 653 base pairs from the amplified sequence. The nucleotide frequency had a strong A/T bias (A = 0.357, T = 0.446, C = 0.104, and G = 0.093). The uncorrected p-distance obtained from pairwise comparison of nucleotide sequences ranged from 0.4% between \textit{Ef.chrysopyga} and \textit{Ef.rufocauda}, and 20% between \textit{Eg.sapphirina} and \textit{El.cingulata} (not shown).

Although 10 sequences were incomplete and 7 sequences taken from Genbank were partially overlapping (550bp), the aligned sequences were sufficient to yield a hypothetical phylogeny (Fig. 2.1). This maximum likelihood tree was obtained using the general time
reversal (GTR) model with gamma distribution ($\alpha = 0.501$), with node support indicated by the
bootstrap value. In the *Euglossa* genus nodes are supported near the tip species, but deeper
nodes in that clade were poorly resolved. The genera *Eulaema* and *Eufriesea* appear to form a
clade, but it is not supported with high bootstrap values. Nonetheless, the topology obtained
with neighbour-joining and Bayesian methods (not shown) always group these two genera. The
*cyt b* information positions *Exaerete* as the sister genus of the *Eulaema-Eufriesea* group.
Finally, the *Euglossa* genus is sister of the *Exaerete* and *Eulaema-Eufriesea* group. Alternative
methods of phylogenetic inference and genetic distance method yielded similar topologies with
nodes with bootstrap values greater than 50% generally conserved (results not shown).

*Morphology*

The relationship between body mass and wing morphology was assessed in 18 species
of Euglossine bees. Body mass ranged from 47 mg (*Eg. sapphirina*) to 1065 mg (*El.
bombiformis*). The relationship between body mass and all variables described below are
expressed as power functions of the form, $Y = aX^b$. Thoracic mass is a constant proportion of
body mass, representing 43% of the total animal mass (C.-A. Darveau and R.K. Suarez,
unpublished). Forewing length and total wing area are related to body mass to the power 0.42
(95% CL: 0.38, 0.46) and 0.87 (95% CL: 0.80, 0.94) respectively (Fig. 2.3A,B). Consequent to
the wing area allometry, the calculated wing loading is higher in larger species, scaling with an
exponent of 0.14 (95% CL: 0.08, 0.21) (Fig. 2.3C). The analyses performed using PIC show
that the body mass effect remains significant using both the phylogeny in Figure 2.1 or
simulated trees (not shown). The allometric exponents obtained using PIC and gradual
evolution from Figure 2.1 were 0.40 (95% CL: 0.34, 0.47), 0.87 (95% CL: 0.72, 1.02) and 0.16
(CL: 0.03, 0.29) for wing length, wing area and wing loading, respectively.
Wingbeat frequency

The effect of body mass on hovering flight wingbeat frequency is presented in Figure 2.4. Frequencies ranged from 86 (Ex. frontalis) to 250 Hz (Eg. heterosticta). Species body mass explains much ($r^2 = 0.86$) of the 3-fold variation in wingbeat frequency, and the scaling exponent of -0.31 (95% CL: -0.24, -0.37) (Fig. 2.4). Wing area and length, however, were related to wingbeat frequency with greater coefficient of determination (Table 2.1).

Analyses of body mass effects on wingbeat frequency performed using PIC are presented in Table 2.1 and Figure 2.5. Using the hypothesised phylogeny in Figure 2.1, the body mass effect remains highly significant using both gradual and speciational evolution (Table 2.1). The exponent obtained is similar to that derived from conventional analysis, with -0.30 (CL: -0.18, -0.43) for gradual and -0.30 (CL: -0.17, -0.43) for speciational evolution. The uncertainty of the hypothesised phylogeny was tested by analysis of the contrasts between body mass and wingbeat frequency using 10,000 different trees obtained from a Bayesian analysis; the frequency distribution clearly shows that wingbeat frequency and body mass evolution are correlated (Fig. 2.5A, B). The average correlation coefficient of the distribution is 0.86 and the values obtained from the cyt b tree in Figure 2.1 are 0.87 and 0.85 for gradual and speciational evolution respectively (Table 2.1 and Fig. 2.5A).

Metabolic rate

The metabolic rates during hovering flight range from 5.4 (Eg. crassipunctata) to 43.2 ml CO$_2$ hr$^{-1}$ (El. bombiformis) and scale against body mass with an exponent of 0.69 (95% CL: 0.60, 0.77) (Fig. 2.6A). Mass-specific rates of CO$_2$ production range from 37.4 to 114.4 ml CO$_2$ hr$^{-1}$ g$^{-1}$, in Ex. frontalis (699 mg) and Eg. hansoni (82 mg), respectively, scaling with an exponent of -0.31 (95% CL: -0.40, -0.23) (Fig. 2.6B). Analysis of the relationships among
mass-specific metabolic rates, wingbeat frequencies, and morphometric parameters reveals that wingbeat frequency mostly explains the variation in metabolic rate, but wing length and area also have a higher coefficient of determination than body mass (Table 2.2).

Incorporating phylogenetic information (Fig. 2.1) into the analysis confirms the relationships presented above, where the mass-specific metabolic rate is accounted for mainly by wingbeat frequency, followed by wing length and area, and then by body mass. Analysing the effect of body mass on mass-specific metabolic rate yields similar exponents for gradual (-0.27 CL: -0.11, -0.42) and speciational (-0.28 CL: -0.14, -0.43) evolution. Testing for phylogeny uncertainty (Fig. 2.5B), shows a significant average correlation coefficient for the distribution of 0.76, while the values obtained using the phylogeny in Figure 2.1 are 0.78 and 0.80 for gradual and speciational evolution, respectively (Table 2.2 and Fig. 2.5B).

Morphology, wingbeat frequency and metabolic rate

To analyse the interrelationship between the body mass, wing morphology, wingbeat frequency and metabolic rate, I first performed stepwise regressions. For wingbeat frequency, wing area was introduced in the regression model, and much of the remaining variation was explained by the wing loading. Moreover, using any of the three variables associated with size (body mass, wing length, wing area), and wing loading, the coefficient of determination of the model was 98%. Stepwise regression analysis of mass-specific metabolic rate introduced wingbeat frequency alone in the model. Performing these analyses using PIC produced the same results qualitatively. In addition, the wingbeat frequency and metabolic rate relationship was analysed for uncertainty (Fig. 2.5C), and a high average correlation coefficient was observed ($r = 0.90$), comparable with those observed with the hypothesised cyt b phylogeny (0.89 and 0.95 for gradual and speciational evolution respectively).
The functional relationships among these variables were further analysed by examining the residuals obtained from the body mass relationship, i.e., as body mass corrected variation. The wingbeat frequency residual variation is greatly explained ($r^2=0.86$) by the wing loading residual variation (Fig. 2.7A). The residuals obtained for wing length and wing area also show strong correlations (not shown), indicating that a species of a given mass with longer wings or wings of greater area will have lower wingbeat frequency, that is, there is a positive correlation between wing loading and wingbeat frequency. The same analyses performed using residuals obtained from the contrast values obtained for gradual and speciational evolution also show strong positive correlation between wingbeat frequency and wing loading (Fig. 2.7B).

The body mass corrected relationship between hovering flight mass-specific metabolic rate and wingbeat frequency is significant and positive (Fig. 2.8A). At one end of the distribution, *Eufriesea pulchra* displays high wingbeat frequency and metabolic rate for its body mass while, at the other end, *Exaerete frontalis* shows low frequency and low metabolic rate for its mass. Analysis of the residuals obtained from PIC analysis confirms this relationship for both models of evolution (Fig. 2.8B). In addition, the conventional statistical analysis performed without *Ef. pulchra* and *Ex. frontalis* yields the same results (not shown).

Analysis of residual variation of metabolic rate also shows that the mass-corrected wing loading is positively correlated with hovering flight energy turnover (Fig. 2.9A). Again, the species *Ef. pulchra* and *Ex. frontalis* are found at either end of the distribution. The relationship still holds upon application of PIC analysis (Fig. 2.9B).
Discussion

Phylogeny of Euglossine bees

The orchid bees (Euglossini) represent the only solitary tribe of the sub-family Apinae, or corbiculate bees, which also includes the eusocial honeybees (Apini), bumblebees (Bombini), and stingless bees (Meliponini). The origin of orchid bees is uncertain, given incongruity between hypothesised morphological and molecular relationships among tribes (Cameron, 2004; Roubik and Hanson, 2004). Within the tribe Euglossini, five genera are found, among which five hypothesised relationships have been proposed over the last 20 years (reviewed by Cameron, 2004). The most recent analysis, using both morphological and molecular data, places the genus *Aglae* closest to the root, and either the genus *Euglossa* or *Exaerete* is proposed as the sister group to *Eulaema-Eufriesea* (Michel-Saltzat et al., 2004).

Prior to the work presented here, there have been no phylogenies available at the species level for orchid bees. The hypothesised phylogeny (Fig. 2.1), which includes 37 species belonging to 4 genera, reveals many species clustered together in groups, consistent with the scheme proposed by Cameron (2004). Nodes near the tip of the tree are supported by high bootstrap values, while the deeper nodes are generally characterized by low bootstrap values (Fig. 2.1). For example, the subgenera classified by Dressier (1978), found in *Euglossa* are sometimes separated in our phylogeny, indicating that they may not derive from a common ancestor (e.g., *Euglossa* VIII, X, XII in Fig. 2.1). One must thus use our hypothesised tree with caution, as a means by which to analyse comparative data and account for uncertainty of tree topology and branch lengths to validate the relationships among these data.
The analysis of flight performance and its determinants reveals linkages between wing morphology and wingbeat frequency during hovering flight. The variation in wingbeat frequency among species is greatly explained by their body mass (Fig. 2.4). This allometric relationship and the scaling exponent of –0.31 (Fig. 2.4) are consistent with results obtained previously by Casey et al. (1985) and, more recently, with results obtained in load-lifting experiments (Dillon and Dudley, 2004). The effect of size on wingbeat frequency can be understood in terms of the resonance properties of the flight apparatus. For a mechanically resonant system such as an asynchronous muscle, the oscillation frequency is inversely proportional to the inertial load on the system, which corresponds to the mass distribution along the wing length (Dudley, 2000). Indeed, reducing inertial load on asynchronous flight muscle by cutting the wings to a shorter length increases the frequency as expected for a resonant system (Sotavalta, 1952). Thus, although body mass and wingbeat frequency are correlated, it is the wing size (length and area) that more directly influences wingbeat frequency. This is supported by the data showing that wing length and wing area are related to wingbeat frequency with greater coefficients of determination than body mass (Table 2.1).

The relationship between wing loading and wingbeat frequency is both negative and weak (Table 2.1). Body mass has differential effects on these two variables, a negative scaling effect on frequency but a positive one for wing loading. Together, these result in the weak negative correlation between wing loading and wingbeat frequency. Byrne et al. (1988) investigated the relationship between body mass, wing loading and wingbeat frequency in insects ranging from 3.3 x 10⁻⁵ to 2.8 grams (Fig. 2.10). They found that wing loading and wingbeat frequency are positively correlated when data are analysed within relatively small ranges of body size. However, when data for the entire range is analysed, wingbeat frequency
and wing loading are no longer correlated. In the present work, I account for the confounding effects of body mass by statistical removal of the effect of body mass on wing loading and wingbeat frequency. This results in a strong positive correlation between these two variables (Fig. 2.7A). Reanalysis of the data of Byrne et al. (1988) along with the Euglossine data reveals the same pattern, with a high correlation coefficient ($r = 0.89$) for the relationship between residuals (Fig. 2.10D). I conclude that in this orchid bee lineage and, possibly, in flying insects in general, wingbeat frequency is strongly related to wing loading, after controlling for body mass covariation.

**Stroke frequency and metabolic rate**

Among aerobic muscles of a given type undergoing prolonged, sustained cycles of contraction and relaxation, operating frequency is the primary determinant of power output (Pennycuick and Rezende, 1984). Because myofibrillar stress and strain are considered to be approximately size independent (Ellington, 1991), the scaling of energetic cost of steady-state flight should be proportional to the operating frequency. The present study shows a strong, positive relationship between wingbeat frequency and metabolic power input ($\dot{V}co_2$) during hovering flight. This strong correlation persists after controlling for both body mass and phylogenetic relatedness. The allometric relationships for wingbeat frequency and mass-specific metabolic rate yield similar exponents (Figs. 2.4 and 2.6) as those obtained by Casey et al. (1985). In the present study, the correlation between metabolic rate and wingbeat frequency residuals lends empirical support for the proposal that wingbeat frequency is the primary determinant of muscle power output and, therefore, metabolic rate during hovering flight.

Dudley (1995), studied hovering flight kinematics in 3 species of orchid bees (Eg. dissimula, Eg. imperialis and El. meriana) flying in hypo-dense mixtures of helium and oxygen
Wingbeat amplitude increased from about 105° in normal air to 140° when flying in heliox. Dillon and Dudley (2004) investigated maximal load-lifting flight capacity in 11 species of orchid bees. They found that orchid bees could sustain about twice their own body mass, and that their load lifting capacity scaled isometrically. While lifting maximal loads, wingbeat frequencies scale allometrically with an exponent similar to that during normal hovering. The wing stroke amplitudes, however, increase to approximately 140° in all species. Thus, interspecifically, stroke amplitude is conserved during normal hovering in ambient air while, intraspecifically, flight power output in response to various imposed loads can be modulated through changes in stroke amplitude. As the bees in the present study were induced to hover in ambient air without added loads, it is reasonable to assume constant stroke amplitude across species.

**PIC and correlated evolution**

Using phylogenetically independent contrasts (PIC) analysis allows incorporation of phylogenetic information into the attempts to account for mechanistic and statistical relationships among the data. The utility of such an approach can be appreciated by considering how body mass of species and correlated characters are strongly influenced by phylogenetic status (Fig. 2.2). An obvious weakness of this approach is the dependence of the outcome on the quality of the phylogeny used. It has been shown that accounting for tree topology and branch length uncertainty can provide some level of confidence in the interpretation of the PIC results (Garland et al., 1993; Martins, 1996; Miles and Dunham, 1993; Housworth and Martins, 2001). The use of Mesquite allowed me to assess the sensitivity of the results to variation in tree topology and branch lengths. By testing the correlated evolution of characters using 10,000 possible phylogenies, including many unlikely scenarios, I show (Fig. 2.5) that the character data are strongly correlated within the vast majority of phylogenetic relationships.
Concluding Remarks

Using hovering insects, I have shown that the functional relationships between animal body mass and energetics can be elucidated. The changes in animal form and proportions with body size were shown to directly influence function and ultimately whole-animal metabolic rate and its scaling. The relationship between body mass and metabolic rate during hovering flight in orchid bees can be understood in terms of the scaling of wing kinematics and morphometric parameters. The body mass effect on metabolism during flight in this lineage occurs through the scaling of wing form and wing loading, which determine the scaling of wingbeat frequency and, therefore, metabolic rate. These results illustrate the intimate relationship between form and function in relation to locomotion, as well as the importance of incorporating an integrative approach in studies of whole-animal metabolic rate scaling.

Recently, models have been proposed to explain the allometric scaling of metabolism based on the assumption that metabolic rates are limited by supply rates via branching (Banavar et al., 1999) or fractal-like (West et al., 1999) distribution systems. During flight in honeybees, metabolism does not appear to be limited by O₂ supply through the tracheal system which, apparently, has considerable excess capacity (Harrison et al., 2001; Joos et al., 1997). Although data in this regard are unavailable for orchid bees, my findings indicate that wing form and kinematics, rather than supply limitations, are the main determinants of flight metabolic rate and its allometric scaling.

During hovering flight in bees, >90% of the oxygen consumed is accounted for by oxidative metabolism in the flight muscles. Within the flight muscles, most of the ATP hydrolysis that occurs is due to actomyosin ATPase activity, which is activated during high rates of cross-bridge cycling (Suarez, 2000). In Chapter 3 of this thesis, I examine the
biochemical correlates of metabolic rate scaling in orchid bees, adding a further dimension to my analysis of the evolution of form and function in this interesting clade.
Table 2.1. Relationship between wingbeat frequency and body morphometries of Euglossine bees using conventional and PIC analyses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Equation</th>
<th>$r^2$</th>
<th>$P$</th>
<th>$b$</th>
<th>$r^2$</th>
<th>$P$</th>
<th>$b$</th>
<th>$r^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_b$ (g)</td>
<td>$106 M_b^{-0.31}$</td>
<td>0.862</td>
<td>&lt;0.001</td>
<td>-0.30 (-0.18, -0.43)</td>
<td>0.756</td>
<td>&lt;0.001</td>
<td>-0.30 (-0.17, -0.43)</td>
<td>0.723</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$R$ (cm)</td>
<td>$188 R^{-0.75}$</td>
<td>0.933</td>
<td>&lt;0.001</td>
<td>-0.80 (-0.59, -1.00)</td>
<td>0.885</td>
<td>&lt;0.001</td>
<td>-0.78 (-0.55, -1.01)</td>
<td>0.853</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$S$ (cm$^2$)</td>
<td>$160 S^{-0.36}$</td>
<td>0.937</td>
<td>&lt;0.001</td>
<td>-0.37 (-0.28, -0.46)</td>
<td>0.899</td>
<td>&lt;0.001</td>
<td>-0.38 (-0.28, -0.48)</td>
<td>0.874</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$P_w$ (g cm$^{-2}$)</td>
<td>$52 P_w^{-0.88}$</td>
<td>0.246</td>
<td>0.036</td>
<td>0.046</td>
<td>NS</td>
<td>0.042</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$N=18$ species for conventional analysis and 11 contrasts for PIC analysis

For gradual evolution, branch lengths from Figure 2.1 were used and for speciational model, branch lengths were set to 1

Symbols: $M_b$, body mass; $R$, wing length; $S$, total wing area; $P_w$, wing loading
Table 2.2. Relationship between mass-specific metabolic rate, wingbeat frequency and body morphometrics of Euglossine bees using conventional and PIC analyses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Conventional</th>
<th>PIC gradual</th>
<th>PIC speciational</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Equation</td>
<td>$r^2$</td>
<td>$P$</td>
</tr>
<tr>
<td>$n$ (Hz)</td>
<td>$0.41 n^{1.00}$</td>
<td>0.887</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$M_b$ (g)</td>
<td>$44 M_b^{-0.31}$</td>
<td>0.797</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$R$ (cm)</td>
<td>$79 R^{-0.76}$</td>
<td>0.862</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$S$ (cm$^2$)</td>
<td>$67 S^{-0.37}$</td>
<td>0.851</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$P_w$ (g cm$^{-2}$)</td>
<td>$19 P_w^{-0.98}$</td>
<td>0.272</td>
<td>0.027</td>
</tr>
</tbody>
</table>

$N=18$ species for conventional analysis and 11 contrasts for PIC analysis
For gradual evolution, branch lengths from Figure 2.1 were used and for speciational model, branch lengths were set to 1
Symbols: $n$, wingbeat frequency; $M_b$, body mass; $R$, wing length; $S$, total wing area; $P_w$, wing loading
Figure 2.1. Phylogenetic tree hypothesised for 36 orchid bee (Apidae: Euglossini) species, based on cyt b partial sequences and inferred using the maximum likelihood method. Node bootstrap support values greater than 50% are shown. Species groupings, based on Cameron (2004), are presented in bold characters.
Figure 2.2. Phylogenetic relationships among 15 of 18 species of orchid bees used for hovering flight measurements. The character values are presented for body mass, $M_b$, wingbeat frequency, $n$, mass-specific metabolic rate, $\dot{V}_{CO_2}$. Sample size, $N$, is identified for each species in parenthesis. Symbols represent different genera.
Figure 2.3. Relationship between body mass and wing morphological characters, forewing length (A), total wing area (B) and calculated wing loading (C). Symbols represent different genera shown in the legend in (A).
Figure 2.4. Relationship between body mass and hovering flight wingbeat frequency. Symbols represent different genera shown in the legend.
Figure 2.5. Relationship between body mass, wingbeat frequency and mass-specific metabolic rate independent contrasts obtained from cyt b phylogeny (Fig. 2.1), and using gradual (filled circles, solid line) and speciational (open circles, dashed line) models of character evolution (A,C,E). These relationships (solid and dashed lines) are superimposed on the distribution of correlation coefficients resulting from analyses performed with 10,000 different trees obtained from a Bayesian analysis (see methods) (B,D,F).
Figure 2.6. Relationship between body mass and hovering flight whole-animal (A) and mass-specific metabolic rate (B). Symbols represent different genera shown in the legend in (A).
Figure 2.7. Correlations between wing loading and wingbeat frequency residuals obtained from the body mass regression (A) ($r^2=0.86$, $F_{1,16}=8.74$, $P=0.0088$) and residuals obtained from independent contrasts (B) for gradual (filled circles, $r^2=0.86$, $F_{1,9}=62.88$, $P<0.0001$) and speciation (open circles, $r^2=0.90$, $F_{1,9}=90.94$, $P<0.0001$) rate of character evolution. Symbols in (A) represent different genera shown in the legend.
Figure 2.8. Correlations between hovering flight wingbeat frequency and mass-specific metabolic rate residuals obtained from the body mass regression (A) ($r^2 = 0.46$, $F_{1,16} = 14.25$, $P = 0.0015$) and residuals obtained from independent contrasts (B) for gradual (filled circles, $r^2 = 0.47$, $F_{1,9} = 8.73$, $P = 0.0144$) and speciation (open circles, $r^2 = 0.70$, $F_{1,9} = 23.73$, $P = 0.0007$) rate of character evolution. Symbols in (A) represent different genera shown in the legend.
Figure 2.9. Correlations between wing loading and mass-specific metabolic rate residuals obtained from the body mass regression (A) ($r^2=0.34$, $F_{1,16}=8.23$, $P=0.0110$) and residuals obtained from independent contrasts (B) for gradual (filled circles, $r^2=0.50$, $F_{1,9}=9.89$, $P=0.0104$) and speciation (open circles, $r^2=0.66$, $F_{1,9}=19.11$, $P=0.0014$) rate of character evolution. Symbols in (A) represent different genera shown in the legend.
Figure 2.10. Relationship between body mass and wing area (A), wing loading (B), and wingbeat frequency (C) in insects ranging from fruit flies to moths (open circles; Byrne et al., 1998) and orchid bees (filled circles). (D) The correlation between wing loading and wingbeat frequency residuals obtained from the body mass regression in B and C.
CHAPTER 3
EVOLUTION OF HOVERING FLIGHT ENERGETICS IN A LINEAGE OF ORCHID BEES
(APIDAE: EUGLOSSINI): DIVERSITY OF METABOLIC PATHWAY DESIGN

Introduction

The diversity of animal locomotion induces a wide range of metabolic energy production rates during activity. For example in mammals, variation in mass-specific metabolic rate during locomotion for sedentary and athletic species of similar size covers a 2.5-fold range, and comparison of all mammalian species shows that mass-specific metabolic rate can range over 100-fold (Weibel, 2000). This wide spectrum of metabolic energy production implies that there could be alterations in metabolic pathway design among species; however, the evolution of muscle cell energy production and pathway design has not yet been explored in the context of locomotory activity. Indeed, only a few comparative studies have incorporated evolutionary approaches into the investigation of metabolic pathway design, and these are studies of temperature adaptation (Pierce and Crawford, 1997). Using analytical approaches developed by evolutionary physiology (Garland and Carter, 1994; Feder et al., 2000) to study metabolic design associated with diversity in locomotory performance provides an opportunity to understand the variation in energy metabolism and its evolution.

Among animals, hovering insect flight muscle has reached the upper-limit of aerobic energy production (Suarez, 2000). In a previous study (Chapter 2), I investigated the evolutionary relationship between the form and function associated with hovering flight in a lineage of orchid bees (Apidea; Euglossini). The correlated evolution found for wing loading, wingbeat frequency and mass-specific metabolic rate identified the consequences of a 20-fold body mass range on flight energetics. Within this group of
bees, there is a 3-fold range in species mass-specific metabolic rate, which provides a means to evaluate the relationship between the evolution of metabolic rate and the design of metabolic pathways.

The use of enzyme maximal velocity, measured in vitro, provides a useful estimate of the maximum capacity for flux (Suarez et al., 1997; Pierce and Crawford, 1997; Fell, 2000), and allows the characterisation of metabolic pathway design. The maximal activity elicited in vitro corresponds to \( V_{\text{max}} = k_{\text{cat}} \times [E] \), where the catalytic efficiency, \( k_{\text{cat}} \), can be considered conserved in interspecific comparison where homologous enzymes functioning at similar temperatures are studied (Suarez, 2000). Thus, \( V_{\text{max}} \) is proportional to the enzyme concentration \([E]\). \( V_{\text{max}} \) not only provides a useful measure to describe enzyme content or expression level, but it can also be compared to the pathway flux rate to provide insight into the relationship between biochemical capacity and physiological load on metabolic pathways (Suarez et al. 1996). Application of such analyses to honeybee flight muscle has illustrated that the high mass-specific metabolic rate in these animals requires high \([E]\) for glycolysis, Krebs cycle and the electron transport chain, and that certain enzymes function close to their \( V_{\text{max}} \) in vivo (Suarez et al. 1996). In addition, Staples and Suarez (1998) showed that although the near-equilibrium glycolytic enzyme phosphoglucoisomerase appears to be present in excess capacity, its activity was close to the calculated value required to yield a net forward flux corresponding to metabolic rate. Given the apparent close match between biochemical capacity and physiological load found for glycolysis in honeybee flight muscle, the question of how metabolic design coevolved with metabolic rate variation within a lineage of asynchronous flyers is of particular interest. In this study, I investigate
the design of flight muscle metabolic pathways in a lineage of orchid bees, by measuring
the activity of selected enzymes involved in fuel delivery, glycolysis, redox balance and
mitochondrial metabolism (Fig. 3.1). The relationship between body mass, hovering
flight metabolic rate and enzyme activity is analysed using the comparative method of
phylogenetically independent contrasts (Felsenstein, 1985). Incorporating this study with
my previous analysis of orchid bee flight energetics evolution (Chapter 2), I identify the
correlated evolution between morphological and biomechanical variables with metabolic
and biochemical variables associated with hovering flight. Because of the close match
between enzyme activity and flux rate in honey bees, and the relationship between
pathway flux rate and metabolic rate, one might predict that the activities of all enzymes
should scale in parallel with metabolic rate. However, if control of metabolic flux is
vested in only a subset of these metabolic enzymes, then one would predict that only the
“rate controlling” enzymes should scale with metabolic rate. At present, our
understanding of biochemical pathways is not sufficiently advanced to assess which of
these alternative hypotheses regarding the control of metabolic flux is more likely to be
accurate. By taking advantage of naturally occurring size variation in orchid bees, and
assessing the data in a phylogenetically appropriate context, I can distinguish between
these possibilities.

Materials and Methods

Orchid bee collection

Measurements of enzyme activity were performed using specimens collected in
2000 and 2001 (Table 3.1). Twenty-eight species were collected in May 2000 at various
sites in Panama by David W. Roubik. Twenty-seven species were collected in June 2001 on Barro Colorado Island, Smithsonian Tropical Research Institute, Panama. Male Euglossini were attracted using cineole, skatol and methyl salicylate (Sigma chemicals), which were found to attract almost all the local species (Dressler, 1982). These chemical baits consist of pure compounds of natural fragrances that male orchid bees collect from various floral and non-floral sources (Roubik and Ackerman, 1987). Specimens were collected using insect nets, collection vials, or by hand when resting on the paper containing the chemicals. Animals were frozen at -80°C until shipment on dry ice to the laboratory for storage at -80°C until enzyme activity measurements were performed. Eight species were collected in June 2001 (Table 3.1) and used for mitochondrial respiration rate measurements performed in the Barro Colorado Island laboratory.

**Enzyme activity measurements**

Using the bees collected in 2000, I measured the activities of hexokinase (HK), phosphofructokinase (PFK), glycerol 3-phosphate dehydrogenase (GPDH), citrate synthase (CS), and cytochrome c oxidase (COX). The bees collected in 2001 were used to measure the activity of trehalase (TR), glycogen phosphorylase (GP) and phosphoglucoisomerase (PGI). Frozen specimens were dissected to isolate the thorax, which was minced with scissors and further homogenized in 19 volumes of ice-cold buffer. All further manipulations were carried out on ice. The homogenization buffer used for HK, PFK, GPDH, CS and COX assays consisted of 25 mM Tris-potassium phosphate pH 7.8 at 4°C, 2 mM ethylene diamine tetra-acetic acid (EDTA), 5 mM dithiothreitol (DTT), 1 mM fructose 6-phosphate, 3.5 mM glucose 6-phosphate and 0.5% (vol/vol) Triton X-100, and for GP, TR and PGI the buffer was composed of 25 mM
Hepes pH 7.3 at 4°C, 2 mM EDTA, 5 mM DTT and 0.5% (vol/vol) Triton X-100. Samples were homogenized using a Polytron (Brinkmann Instruments), using three 10 seconds passes with 30 seconds rest in between. Samples were also sonicated using a Micro Ultrasonic Cell Disrupter (Kontes), again using three 10 seconds passes and 30 seconds rest in between. Crude homogenates were centrifuged (Jouan, MR 1812) for 5 minutes at 10 000 RPM (8,000 g) at 4°C, and the supernatant was used for assays.

Enzyme activities were measured in duplicate using a Perkin-Elmer UV-Visible spectrophotometer (model Lambda 2), serviced with a circulating water bath maintaining the cuvettes temperature at 37°C. The HK, PFK, G3PDH, PGI, TR, GP reactions were monitored by following the rate of appearance or disappearance of reduced nicotinamide adenine dinucleotide (NADH) or NADPH at a wavelength of 340 nm, and the millimolar extinction coefficient (ε) of 6.22 was used for calculation of activity. The CS reaction monitored 5,5’ dithiobis-(2-nitrobenzoic acid) (DTNB) at 412 nm and ε = 13.6 was used. The COX reaction monitored oxidized cytochrome c at 550 nm and used ε = 29.5. For the HK and PFK reactions, control rates obtained in the absence of substrate (approximately 5-10% of the activity) were subtracted from rates obtained with all substrates present. The enzyme activity, expressed as international units (U = μmol of substrate converted to product per minute), was ensured to be linear and was calculated for the first 2 or 3 minutes of the reactions.

Enzyme assay conditions were optimized to elicit maximal reaction velocity at 37°C. HK: 50 mM Hepes pH 7.0, 5 mM D-glucose (omitted from control), 4 mM adenosine triphosphate, 10 mM magnesium chloride, 100 mM potassium chloride, 0.5
mM NADP, 5 mM DTT, 1 U glucose 6-phosphate dehydrogenase. PFK : 50 mM Tris-
HCl pH 8.0, 5 mM fructose 6-phosphate (ommitted from control), 10 mM magnesium
chloride, 100 mM potassium chloride, 2 mM adenosine triphosphate, 0.15 mM
Nicotinamide Adenine Dinucleotide H, 0.01 mM fructose 2,6-bisphosphate, 5 mM DTT,
1 U aldolase, 5 U triosephosphate isomerase, 5 U α-glycerophosphate dehydrogenase.
G3PDH : 50 mM imidazol pH 7.0, 1 mM dihydroxyacetonephosphate, 0.15 mM NADH.
CS : 50 mM Tris-HCl pH 8.0, 0.5 mM oxaloacetate, 0.3 mM acetyl-CoA, 0.1 mM
DTNB. COX : 50 mM potassium phosphate pH 7.5, 0.05 mM reduced cytochrome c.
TR : 50 mM potassium phosphate pH 6.6, 1.1 mM magnesium chloride, 0.5 mM NADP,
1.1 mM ATP, 10 mM trehalose, 2.5 U of hexokinase and glucose 6-phosphate
dehydrogenase. GP : 100 mM potassium phosphate pH 7.4, 2 mg/ml glycogen, 0.5 mM
NADP, 4 µM glucose 1,6-biphosphate, 2 mM adenosine monophosphate, 10 mM
magnesium chloride, 10 U phosphoglucomutase and 2.5 U glucose 6-phosphate
dehydrogenase. PGI : 50 mM Tris-HCl pH 8.0, 0.5 mM fructose 6-phosphate, 0.5 mM
NADP, 2.5 U glucose 6-phosphate dehydrogenase. All chemicals used were obtained
from Sigma chemicals.

Homogenate respiration rate measurements

I used a thoracic homogenate preparation to estimate mitochondrial respiration
rate, which was performed on eight species (Table 3.1). The use of isolated mitochondria
was impractical due to the large number of individuals required to isolate flight muscle
mitochondria (approximately 2 grams of bee thoraces). The method described below was
shown to exhibit reproducible maximum respiration rates. In addition, the ratio between
homogenate respiration rate and cytochrome oxidase respiration rate was 1/10 (data not shown), which was similar to the ratio obtained using isolated mitochondria preparation (R.K. Suarez, unpublished; C.-A. Darveau and P.U. Blier, unpublished). Homogenate respiration was thus used as an index of mitochondrial maximum respiration rate, and is further referred to as mitochondrial respiration rate.

After the bees were captured in the field, they were stored in the refrigerator at 4°C until measurements were performed. Before preparing the thorax for homogenization, the individual bee had to be warmed up until leg movements were noticeable. Skipping the rewarming procedure seemed to cause inactivity of the mitochondria, as there was no detectable respiration in preparations made from cold bees. After the thorax was dissected from the insect, further manipulations were performed on ice. Individual thoraces were minced with scissors and homogenized in 19 volumes of ice-cold 10 mM Tris pH 7.4 at room temperature, 1mM EGTA and 250mM sucrose, using a single slow speed 10 second homogenization using a Polytron (Brinkmann Instruments).

The rate of mitochondrial respiration was measured at 37°C in a 1.6 ml water-jacketed Gilson glass chamber, equipped with a clark type electrode (YSI) to monitor changes in dissolved oxygen content. The assay buffer (10 mM Tris pH 7.4 at 20°C, 1mM EGTA, 25mM KH₂PO₄ and 154mM KCl) was equilibrated with air before the measurements, and oxygen content was assumed to be of 406 nmol O ml⁻¹ in these conditions, according to Reynafarje et al. (1985). After the addition of 50 µl of homogenate, 10 µl of 1 M pyruvate and proline were added and respiration was initiated.
by adding 20 µl of 40 mM adenosine diphosphate. Measurements were performed in triplicate and expressed as µmol O₂ min⁻¹ g⁻¹. Data were acquired and rate analyzed using Datacan (Sable Systems).

Data analysis

All data are presented as species mean values with standard deviation, but all analyses presented below were also performed using individual data points. Species samples were randomised to reduce the effect of assay date on species enzyme activity mean estimates and assay date was also included in statistical analyses. The effect of body mass on the different variables was tested using log transformed data to linearise the relationship, expressed as Y = aXᵇ.

Analysis of enzyme fractional velocity was conducted by calculating the in vivo pathway flux rate, divided by the maximal enzyme activity, expressed in µmol min⁻¹ g⁻¹ thorax, times 100 (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 analysed using phylogenetically independent contrasts (Felsenstein, 1985) using the PDAP module (Midford et al., 2003) included in Mesquite (Maddison and Maddison, 2004). I used the hypothesised phylogeny based on cytochrome b sequence from my previous work (Chapter 2; Fig. 2.1) 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. I ensured the contrasts were adequately standardised by plotting the absolute value of standardised independent contrasts against their standard deviation (Garland et al., 1992). I also tested for branch length and
topology uncertainty by performing simulations using 10,000 possible trees and reported
the frequency distribution of independent contrast correlation coefficient, using Mesquite.

Results

Enzyme activity

For both GP and TR, there was a significant decrease in enzyme activity with
increasing body mass (Fig. 3.2A,B). These power relationships lead to an approximately
1.5-fold difference in activities for both enzymes. The smallest 50 mg bee, *Euglossa sapphirina*,
had an average activity of 25.7 U g$^{-1}$ thorax for TR and 14.7 U g$^{-1}$ thorax for
GP, while the mean activity of the largest 1 g bee, *Eufriesea ornata*, was 15.16 and 7.35
U g$^{-1}$ thorax for TR and GP respectively. This effect of body mass on enzyme activity
remained significant when using species mean values or individual data points.

The activities of enzymes catalyzing three consecutive steps initiating glycolysis
were measured. HK activity follows a distinct relationship with species body mass, which
can be described by the power function, $HK = 26.9M_b^{-0.33}$ (Fig. 3.3A). This allometric
relationship led to about 2.5-fold difference in enzyme activity between the smallest and
largest species. The activity of the regulatory enzyme PFK was independent of body
mass, with an overall mean of $108.2 \pm 18.6$ U g$^{-1}$ thorax (Fig. 3.3B). The activities of the
near-equilibrium enzymes PGI and GPDH were also independent of body mass, with an
overall activity mean value $345.6 \pm 51$ and $616.9 \pm 133.1$ U g$^{-1}$ thorax respectively (Fig.
3.3C,D).
The Krebs cycle enzyme CS was positively related to body mass (Fig. 3.4A). Two clusters of data points are apparent, grouped by genus. The activity of the electron transport chain enzyme, COX, was independent of species body mass (Fig. 3.4B), but a significant negative relationship was observed when individual data points were considered in the analysis (Fig. 3.4C).

**Mitochondrial respiration**

Mitochondrial respiration rate was measured in 8 species of orchid bees, using a crude homogenate technique to compare species estimates of maximal mitochondrial respiration rate. Using species mean values (Fig. 3.4D), there was no significant relationship between body mass and mass-specific mitochondrial respiration rate, however, a significant increase was detected using individual data points (Fig. 3.4E).

**Flight energetics and metabolic design**

I correlated the enzyme data with the mass-specific metabolic rates of 14 species for which I had data for both parameters (Chapter 2). The activity of HK, COX and GP were positively correlated with mass-specific metabolic rate (Fig. 3.5A-C), with the exponent 0.97, 0.47 and 0.36 respectively. The activity of HK scaled with an exponent value ($b = -0.33$) similar to that obtained for mass-specific metabolic rate and wingbeat frequency scaling ($b = -0.31$) during hovering flight (Fig. 3.6). Residuals obtained from body mass relationships were analysed and a significant positive correlation was detected (Fig. 3.7A).
From the hovering flight mass-specific metabolic rate, I could estimate the in vivo pathway flux rates. When converted to the same units, the ratio of pathway flux rate to enzyme maximal flux capacity (v/Vmax x 100) indicates the fractional velocity or %Vmax, at which the enzyme functions (Suarez et al., 1996). Figure 3.8 presents the relationship between body mass and fractional velocity of glycolytic and mitochondrial enzymes. This analysis shows that only for HK the fractional velocity is conserved, with an average of 20.4 ± 3.5 %. In all other cases, there is a significant decrease in fractional velocity with increasing body mass.

*Phylogenetically independent contrasts*

Phylogenetic analysis was performed using a hypothetical tree based on cyt b gene partial sequence (Chapter 2, Fig. 2.1), but to account for topology and branch length uncertainty, I implemented the analysis with distribution of correlation coefficient obtained from 10,000 possible trees generated from previous Bayesian analysis (see Chapter 2). Figure 3.9A presents the significant relationships between independent contrasts in body mass and HK activity obtained for gradual and speciational model of character evolution using cyt b phylogeny (Fig. 2.1). These correlation coefficients were superimposed on the distribution of correlation coefficients obtained using an array of 10,000 phylogenies (Fig. 3.9B). The same treatment of the data also showed a significant effect of body mass on CS and GP where almost all relationships are significant (Fig. 3.9C,D), whereas for TR the correlations obtained from cyt b phylogeny are significant, but many correlations of the distribution are non-significant (Fig. 3.9E).
The correlation between hovering flight mass-specific metabolic rate and enzyme activity was also analysed using both \textit{cyt b} phylogeny and 10,000 trees obtained from Bayesian analysis (Chapter 2). For HK, the relationship was significant using all phylogenies (Fig. 3.10A). For COX and GP activity, most relationships were non-significant (Fig. 3.10B,C).

**Discussion**

*Metabolic design*

The metabolic organisation of insect flight muscle has been investigated using broad phylogenetic comparisons, contrasting invertebrate and vertebrate muscle tissues (Crabtree and Newsholme, 1972; Suarez et al. 1997). Such comparisons described the general metabolic organisation of hovering insect flight muscles, however, how metabolic pathways coevolved with variation in flight metabolic rate within a group of asynchronous flyers has not previously been investigated. Nonetheless, comparison of these different evolutionary time scales could allow evaluation of some potential targets of energy metabolic pathways that coevolve with metabolic rate. The study of Crabtree and Newsholme (1972) showed that hymenopteran and dipteran flight muscles possess high activities of the regulatory glycolytic enzymes HK and PFK, which suggests that both enzymes are likely to be involved in metabolic rate variation and evolution. Furthermore, the flight muscles of these flying insects have high activity of GPDH, which is involved in the maintenance of redox balance, and low activity of GP. These enzyme levels agree with the suggestion that asynchronous flying insects power flight using only carbohydrates (Saktor, 1965; Weis-Fogh, 1967; Rothe and Natchigall, 1989). Also, the
low glycogenolytic capacity and glycogen reserves support that glycogen is probably not sufficient to power flight (Crabtree and Newsholme, 1972; Panzenbock and Crailsheim, 1997). Finally, Suarez et al. (1996) described asynchronous flyers as having high enzymatic flux capacities for glycolysis, Krebs cycle and electron transport chain. Using orchid bees, evolution of metabolic design associated with metabolic rate variation can be evaluated at a finer evolutionary time scale than comparisons so far examined.

Metabolic diversity

Diversity and allometric scaling of metabolic rate during hovering flight in the lineage of orchid bees was first described by Casey et al. (1985, 1992). In a subsequent study, I further showed the correlated evolution of wing morphology, kinematics and flight muscle energetics (Chapter 2). In this group of orchid bees, flight mass-specific metabolic rate variation is strongly related to species body mass (Fig. 3.6). Detailed analysis of this scaling relationship revealed that body mass appears to be a proxy for wing size, which was revealed by the strong correlated evolution of wing loading and wingbeat frequency. This relationship is important in understanding the links between form and function associated with flight because wingbeat frequency represents the functional load on orchid bee flight muscle. The wingbeat frequency corresponds to the flight muscle contraction frequency, which is directly related to mass-specific metabolic rate (Chapter 2 and Fig. 3.6). Furthermore, the relationship between wing form, kinematics and mass-specific metabolic rate was significant after controlling for phylogenetic relatedness and body mass covariation, which support the functional relationship among those variables. This description of flight metabolic rate diversity in
orchid bees provides a framework to understand species size effects on physiology and metabolism.

Given the effect of body size on metabolic rate during hovering flight in orchid bees, one might expect body size to also influence the metabolic design of flight muscle cells, as an approximately 3-fold range in mass-specific metabolic rate was found across the range of body mass covered by this group of bees (Fig. 3.6). A direct relationship between flight muscle metabolic rate and enzyme activity would yield an approximately 3-fold lower activity for large orchid bee species. It is surprising that no decrease in activity was detected for the mitochondrial enzyme CS and COX, as previous work from Casey et al. (1992) suggested a decrease in mitochondrial volume with increasing size in orchid bees, and studies on mitochondrial enzyme activity scaling suggested a decrease in activity with increasing size in mammals (Emmett and Hochachka, 1981) and fish (Somero and Childress, 1980). 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 for a given body mass (i.e. the mitochondrial volume of a 150 mg species ranged from 35 to 43%). Such variation in mitochondrial content and the data provided in the present study for CS, COX, and mitochondrial respiration estimates (Fig. 3.4.A-E), suggests that mitochondrial machinery is not quantitatively adjusted to metabolic rate variation in orchid bee flight muscle. Instead, I observed variable patterns, where CS activity increased in larger species and the same tendency was observed for mitochondrial respiration estimates, while COX activity was quite variable and independent of size. Such data are inconsistent with those obtained in mammals and fish, which suggest that mitochondrial machinery correlates with mass-specific metabolic rate
and follows similar scaling patterns. Possibly, the metabolic organization of bee flight muscle could explain the absence of such relationship, as aerobic metabolic pathways not only involve Krebs cycle and electron transport chain, but also glycolysis which is an obligatory aerobic pathway in such muscle (Hochachka and Somero, 1984).

The most striking result of the present study is the distinct scaling relationship observed for HK, which shows parallel scaling with wingbeat frequency and mass-specific metabolic rate during hovering flight (Fig. 3.6). The similar scaling relationship between HK and metabolic rate suggests a functional connection between this enzymatic step and overall aerobic metabolic pathway flux. Interestingly, the study of glycolytic enzyme scaling relationships have thus far shown positive scaling, as the mass-specific activity increases with body mass in mammals (Emmett and Hochachka, 1981), fish (Somero and Childress, 1980), reptiles (Baldwin et al., 1995), amphibians (Miller et al., 1993) and crustaceans (Baldwin et al., 1999). Such a pattern, however, was associated with the function of the pathway in those organisms, which largely relies on anaerobic glycolysis to perform burst locomotion. The mass-specific cost of burst locomotion increases with species body mass, thus glycolytic enzyme activity scales positively to accomplish such activity (Somero and Childress, 1980). Insect flight muscles are strictly aerobic, and the negative scaling observed for HK agrees with the pathway function, geared for aerobic metabolism and providing substrate for oxidative phosphorylation. Scaling relationships of enzyme activity appear intimately linked to pathway function, however only for selected enzymes.
Pathway flux and enzyme activities

Using this lineage of orchid bees, one can examine the correlated evolution of metabolic pathway flux rate and enzyme activity. A recent discussion of the subject by Fell (2000), points out that the available models of metabolic pathway regulation would predict distinct patterns of gene expression. Models implying single rate limiting steps would show the activity of one or a few selected enzymes to correlate with pathway flux rate variation. Such models have been seriously questioned by metabolic control theory (discussed below), which proposed the alternative hypothesis that all or most enzyme activities should correlate with metabolic flux rate (Fell, 2000). Furthermore, studies have also suggested that enzyme properties, such as distance from equilibrium, would also be involved in determining how enzyme activity correlates with pathway flux. Based on empirical studies, Hochachka et al. (1998) suggested that near-equilibrium enzymes would undergo the least amount of change with changes in pathway flux rate, as the variation in flux through such enzymes can be accounted for by modulators. On the other hand, Fell (2000) showed that empirical studies have demonstrated the opposite, where near-equilibrium enzymes showed the most change with pathway flux. Clearly, no obvious conceptual framework allows the a priori prediction of the correlated evolution between pathway and enzymatic flux.

A closer examination of metabolic control analysis and its application can provide additional predictions. Based on theoretical arguments, Fell and Thomas (1995) argued that the classic model of rate limiting enzymes would induce changes in the concentration of pathway intermediates, instead of overall flux through the pathway. Their alternative involves multi-site modulation of metabolic pathways, where control is shared among
many steps along pathways. Application of metabolic control analysis to study glycolysis in various tissue types showed that the distribution of control varies depending on the metabolic state under investigation. Kashiwaya et al. (1998) showed that much of the control in rat heart fuelling on glucose resides in the hexokinase and glucose transport steps, which was further shown to apply for skeletal muscle (Fueger et al, 2004), and other tissue types (Puijaner et al. 1997; Whitesell et al 2003). Thus, much of the control of glycolysis in situations or tissues where only hexose is used as a fuel source appears to be found at the hexokinase step. In light of such mechanism, variation in glycolytic pathway flux in orchid bee flight muscle would be predicted to act on major controllers of glycolysis such as HK, as it is observed in the present study (Fig. 3.6, 3.10). Therefore, no simple property, like distance from equilibrium (Hochachka et al. 1998; Fell, 2000), can explain why the expression of specific enzyme is altered. Instead, considerations of enzyme function and control in the context of the entire metabolic pathway could provide the basis for adjustments and evolution of enzyme expression.

One way to quantify the degree and the type of regulation of enzymatic reactions in a pathway was developed by ter Kuile and Westerhoff (2001). These authors looked at the intraspecific adjustments in expression of glycolytic enzymes in 3 species of *Trypanosoma*, grown in varying chemostat cultures. These authors observed that only one or two enzymes changed in activity to maintain their relative proportion with changes in glycolytic flux rate, while most others did not change in activity. These authors stressed the importance of metabolic changes, including changes in substrates, products and modifiers of the reactions rather than changes in enzyme concentration itself. The changes in enzyme concentration, referred to as hierarchical changes, are possible via
alteration in mRNA sequestration and intracellular localisation, or rates of transcription, translation and degradation (ter Kuile and Westerhoff, 2001). These authors quantified the degree of hierarchical changes using the hierarchical regulation coefficient, $\rho_h$, which can be represented graphically as the slope of the relationship between pathway flux rate and enzyme activity, presented on log-log plot. The changes in flux through a given step is accounted for solely by metabolic regulation when no relationship is found between the enzyme activity and pathway flux rate, $-0.2 < \rho_h < 0.2$, but it is under complete hierarchical regulation when the activity is directly related to pathway flux rate, $0.8 < \rho_h < 1.2$. Additionally, an intermediate relationship was observed when $0.2 < \rho_h < 0.8$, where a shared metabolic and hierarchical regulation was observed. My study shows complete hierarchical regulation of HK with $\rho_h = 0.98$ (Fig. 3.5A), and an apparent shared control of COX and GP where $\rho_h = 0.46$ and 0.36 respectively (Fig. 3.5B, C), however, the relationship remains significant only for HK when controlling for phylogeny (Fig. 3.9). This interspecific comparison agrees with ter Kuile and Westerhoff (2001) intraspecific observations, where metabolic regulation can account for most changes in flux through the majority of the linked steps in energy metabolism pathways. Using this concept, one can identify which type of regulation explains flux rate variation through a given step of a pathway, and quantify it using metabolic and hierarchical coefficients.

*Metabolic regulation and evolutionary design*

From a mechanistic point of view, HK is known to be a regulator of glycolysis in many tissue types (Cardenas et al., 1998), including insect flight muscle (Saktor, 1975; Storey, 1980). The importance of HK as a regulator of glycolysis might explain the conservation of HK fractional velocity. It can be seen in Figure 3.8 that HK %Vmax is
conserved across orchid bee species, and it can be suggested that such a property might have to be conserved to maintain regulatory properties of the enzyme. In a paper by Suarez et al. (1996), it was suggested that PFK was kept optimally poised in vivo for regulation by allosteric modulators, as %Vmax was maintained close to 50%. The present study shows that PFK %Vmax decreases with increasing body size (from 22 to 4 % Vmax) while HK fractional velocity is maintained at close to 20% (Fig. 3.8B). The conserved %Vmax found for HK suggests that this specific step might be an important regulator of hovering insect flight muscle glycolysis, as suggested by Crabtree and Newsholme (1972). The need for hierarchical regulation of this specific step might involve the conservation of regulatory power over the pathway flux rate. In honeybee flight muscle, the products of the reaction are inhibitory. ADP is a competitive inhibitor with respect to ATP, and glucose 6-phosphate is non-competitive with glucose (Saktor, 1975). Staples and Suarez (1997) measured glucose 6-phosphate concentration in freeze-clamped honeybee flight muscle at rest and during flight, and showed a greater concentration during flight. Based only on product concentration, a greater inhibition of HK would be predicted during flight, however, changes in concentration of other regulators (ADP, Pi) or conditions (temperature, pH) can act in concert on HK and modify its regulatory properties (Storey, 1980; Scaraffia and Gerez de Burgos, 2000). Further studies of hymenoptera HK regulation are required to evaluate the suggestion that regulatory properties are conserved with constant %Vmax.

Previous analysis of metabolic pathway design evolution has suggested that there are evolutionary adjustments in the activity of multiple enzymes along the glycolytic pathway. Pierce and Crawford (1997), examined the correlated evolution of expression of
glycolytic enzymes among fish species adapted to various environmental temperatures, and found that the activity of 3 glycolytic enzymes, the far from equilibrium enzyme pyruvate kinase, the near-equilibrium enzymes glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase, was correlated with mean annual temperature. These authors suggested that this evolutionary analysis of glycolytic enzyme expression supports the concept of metabolic control theory, where multiple-steps along biochemical pathways control overall pathway flux. Although I reach similar conclusions, the study of Pierce and Crawford (1997) does not provide mechanistic reasoning for the coordinated adjustment of several enzymes. Using insect flight muscle, the variation in pathway maximal flux rate can easily be quantified using flight metabolic rate measurements, and asynchronous flight muscle design constrained energy metabolism pathway to aerobic glycolysis as the only pathway supplying mitochondrial metabolism. This evolutionary analysis of metabolic pathway design thus provides an example of metabolic diversity, but also provides testable mechanistic hypotheses based on evolutionary patterns. Further analysis linking patterns of gene expression evolution with functional consequences on whole metabolic pathway is the next step for such analysis.

Concluding remarks

In my previous analysis of hovering flight metabolic rate evolution, I established a link between form and function of the flight apparatus. Here I extend this analysis and show the biochemical correlates of metabolic rate evolution. Combining the two studies, one can connect the animal's morphological and molecular design associated with hovering flight. Over the range of body mass found in this group of bees, wing size and wing loading impact the wingbeat frequency during hovering flight, which directly sets
the physiological load on flight muscle. Analysis of metabolic pathway design showed the correlated evolution of the glycolytic enzyme hexokinase, a regulatory enzyme of glycolysis. This series of studies, therefore, shows the correlated evolution of characters related to hovering flight, from morphological to biochemical traits. This study shows the importance of integrative and mechanistic considerations in the study of metabolic rate evolution and its relationship with species body mass.

The evolution of metabolic pathway design cannot be predicted from simple biochemical properties of individual enzymes. For example, the generalisation that non-equilibrium enzymes having a regulatory role are the targeted sites of evolution does not appear to apply in the present hovering insect flight muscle model. Instead, considering the properties of the pathway as a whole appears to provide the basis for evolutionarily adjustments correlated with pathway flux rate diversity and evolution. Indeed, mechanistic studies consistently showed that in aerobic tissues fuelling strictly on glucose, most of the control is found at the hexokinase and glucose transport step (Puijaner et al., 1997; Kashiwaya et al., 1998; Whitesell et al., 2003; Fueger et al., 2004). Our understanding of metabolism diversity should benefit from such an approach, as both mechanistic perspectives and evolutionary history can be integrated.

Authors of recent studies investigating the effect of body mass on metabolism suggest that there is no reason to believe that metabolic machinery (such as enzyme activity) should scale with species body mass (Banavar et al., 2002). Instead, metabolic rate was proposed to be a consequence of delivery systems limitations (Banavar et al, 1999, West et al., 1999). No such limitation of the tracheal system has been demonstrated in bees (Harrison et al., 2001; Joos et al., 1997) and the present study shows that there is
correlated evolution of body mass, wing morphology, flight metabolic rate and molecular machinery. It appears that biomechanical consideration of flight energetics can adequately explain flight metabolic rate scaling in this group of bees, which is an alternative view to the increasingly popular theories involving distribution network limitation. Of course the mechanisms and processes involved during flight metabolic rate differ from resting metabolic rate, which is the metabolic state usually 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 the correlation or correlated evolution between resting energy expenditure and maximal metabolic rate associated with activity such as locomotion or thermoregulation. Thus, integrative analysis of body mass effect on an animal's energetics should ideally combine mechanistic and evolutionary perspectives, combining micro and macrophysiological studies (Chown et al., 2004).
Table 3.1. List of orchid bee species collected for measurements of enzyme activity and mitochondrial respiration rate.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species (enzymes 2000)</th>
<th>Species (enzymes 2001)</th>
<th>Species (mitochondria)</th>
</tr>
</thead>
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<td>anisochlora (7)</td>
<td>chrysopyga (3)</td>
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<td>ornata (3)</td>
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</tr>
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<td></td>
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<td>cingulata (3)</td>
<td>cingulata (1)</td>
</tr>
<tr>
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<td>nigrita (7)</td>
<td>nigrita (3)</td>
</tr>
<tr>
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<td>frontalis (5)</td>
<td>frontalis (1)</td>
<td></td>
</tr>
<tr>
<td>Exaerete</td>
<td>smaragdina (1)</td>
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</table>
Figure 3.1. Location of enzymes measured as indicator of maximum flux capacities for fuel delivery (trehalase, glycogen phosphorylase), glycolysis (hexokinase, phosphoglucoisomerase, phosphofructokinase), redox balance (glycerol 3-phosphate dehydrogenase), Krebs cycle (citrate synthase) and electron transport chain (cytochrome c oxidase).
Figure 3.2. Relationship between body mass and (A) trehalase and (B) glycogen phosphorylase activity measured in 27 species of orchid bees. Symbols represent different genera shown in the legend in (A).
Figure 3.3. Relationship between body mass and (A) hexokinase, (B) phosphoglucoisomerase, (C) phosphofructokinase, and (D) glycerol 3-phosphate dehydrogenase activity measured in 28 species of orchid bees. Symbols represent different genera shown in the legend in (A).
Figure 3.4. Relationship between body mass and (A) citrate synthase and (B) cytochrome c oxidase activity measured in 28 species of orchid bees. For COX, a significant ($r^2 = 0.09; P < 0.01$) negative relationship was observed when individual data points were used (C). The relationship between body mass and mitochondrial respiration rate was measured in 8 species (D) and was found significant ($r^2 = 0.42; P < 0.001$) only when individual data points were included (E). Symbols represent different genera shown in the legend in (A).
Figure 3.5. Relationship between hovering flight mass-specific metabolic rate and the activity of (A) hexokinase, (B) cytochrome c oxidase and (C) glycogen phosphorylase. Symbols represent different genera shown in the legend in (A).
Figure 3.6. 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 (long dash: $V_{\text{CO}_2} = 44 \ M_b^{-0.31}, r^2 = 0.80$), and hexokinase activity (short dash: $27 \ M_b^{-0.33}, r^2 = 0.82$). Symbols represent different genera shown in the legend.
Figure 3.7. 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 relationships in Figure 3.6 (A). The same relationship (non-significant in both cases) is presented for independent contrast obtained from the cyt $b$ phylogeny using gradual (filled circles) and speciational (open circles) model of character evolution (B). Symbols in (A) represent different genera shown in the legend.
Figure 3.8. Relationship between body mass and the fractional velocity (%Vmax) of (A) glycogen phosphorylase (open triangles) and trehalase (filled triangles), (B) the glycolytic enzyme hexokinase (open circles), phosphoglucoisomerase (open triangles), phosphofructokinase (filled circles) and glycerol 3-phosphate dehydrogenase (filled triangles), and (C) the mitochondrial enzymes citrate synthase (open squares) and cytochrome c oxidase (filled squares).
Figure 3.9. Correlation coefficients between the independent contrasts in body mass and enzyme activities of hexokinase (A,B), citrate synthase (C), glycogen phosphorylase (D) and trehalase (E). Solid lines represent analyses performed using the gradual model of character evolution while dashed 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 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 $\alpha = 0.05$ level (2-tailed) is 0.576 for HK, GP and TR (df = 10) and 0.602 for CS (df = 9).
Figure 3.10. correlation coefficient between the independent contrasts in hovering flight mass-specific metabolic rate and the activity of (A) hexokinase, (B) cytochrome c oxidase and (C) glycogen phosphorylase. The distribution of correlation coefficients results from analyses performed with 10,000 different trees (see methods). The correlation coefficient obtained from cyt b sequence information using a gradual (solid lines) and speciational (dashed 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 area represent non-significant relationships given that the critical values of the correlation coefficient $r$ for significance at the $\alpha = 0.05$ level (2-tailed) is 0.576 (d.f. = 10).
CHAPTER 4
ALLOMETRIC CASCADE AS A MULTIPLE-CAUSE EXPLANATION OF BODY MASS EFFECTS ON METABOLISM

Introduction

The research presented in Chapters 2 and 3 exploits the effect of body mass on animal energetics to test hypotheses concerning the correlated evolution of form and function associated with insect hovering flight. From these data, it is clear that flight metabolic rate does not scale linearly with body mass. Indeed, for more than a century, biologists have known that metabolic rates of organisms follow an allometric, rather than an isometric (1:1), relationship with body mass. The well known power function of metabolic rate (MR) scaling is expressed as

\[ MR = aM_b^b \] (1)

where \( a \) corresponds to a proportionality coefficient (or intercept in linearized log-log plot), \( M_b \) is body mass, and \( b \) is the scaling exponent (or slope of the log transformed data). Rubner (1883), was the first to demonstrate such a scaling relationship, showing that in mammals basal metabolic rate (BMR) scaled with an exponent of 2/3. Rubner suggested that the scaling for the cost of maintenance in mammals would be proportional to the heat loss through body surface area, hence the observed surface area to volume ratio or 2/3 exponent. The conundrum arose when Kleiber (1932), expanded the number of mammalian species examined to include a larger range of body masses, and observed a 3/4 rather than a 2/3 exponent. The difference in the numerical value of the scaling exponent had a profound effect on this field of research because it became the basis for theories explaining the phenomenon. Recently Kleiber’s relationship has been treated as a new law of life, the 3/4 power “law” (West et al., 1997, 1999, 2000) that is regarded as one of the bases for the “metabolic theory of ecology.”

Recent theories based on distribution networks in living systems (West et al., 1997, 1999, 2000; Banavar et al., 1999, 2002) have generated much attention. West et al. (1997, 1999,
2000), proposed that the fractal geometry of any distribution network (circulation of vertebrates, tracheal system of insects, xylem-phloem of plants, virtual fractal networks within cells) would predict a 3/4 rather than a 2/3 scaling exponent. The breakthrough was the demonstration that fractal networks introduce a fourth dimension; the quarter power exponent can be derived from such properties. Banavar et al. (1999), however, showed that fractal geometry is not required, and that the properties of efficient transportation networks alone can explain the 3/4 scaling. Both theories assume that physiological systems involved in material supply are the basis for allometric scaling in biology. My own observation that insect flight metabolic rate scales allometrically (Chapters 2,3) despite no apparent limitation of the tracheal delivery system (Joos et al., 1997; Harrison et al., 2001), combined with the large body of literature available for mammals, motivated me to investigate the basis of this apparently fundamental biological relationship.

Variations in allometric exponent and intercept

The large amount of data now available facilitates the reexamination of the allometric scaling of BMR across a wide spectrum of organisms. Thus, recent data compilations (such as Dodds et al. (2001), re-assessing Kleiber's data; Heusner (1991), on 391 species of mammals; Bennett and Harvey (1987), on 398 species of birds; and Bartels (1982), on 85 mammals) find surprisingly little evidence favouring a 3/4 rather than a 2/3 scaling exponent. This is also true for analyses incorporating mammalian phylogenetic information, where phylogenies based in morphological characters support the 3/4 exponent, while more recent molecular phylogenies tend to support the 2/3 exponent (Symonds and Elgar, 2002). These analyses of large databases demonstrate extensive variability in the $b$ exponents, that are sometimes around 0.75, but more often are lower (Heusner, 1991; Bennett and Harvey, 1987). These also vary depending on the range of size examined (Dodds et al., 2001; Heusner, 1991; Makarieva et al., 2003). When
Lovegrove (2000) surveyed literature data on the BMR of 487 mammal species, he found that the data clustered into subgroups according to their zoogeographic region and according to environment (desert vs mesic). Not only was great variability of the exponent once again noted, so also was there variability of the proportionality coefficient, $a$, of equation (1). This study emphasised the influence of an unpredictable (desert) environment in reducing the $a$ value, in other words in reducing the metabolic “base line”. The variation in metabolic rate was also shown to be size-dependent, in this case observing a “constrained body mass” of about 350 g where variance of BMR is minimal and increasing in smaller and larger species.

More recently, White and Seymour (2004), compiled the largest data set so far presented for mammals, and show a scaling exponent of 2/3 in over 500 mammalian species. Nonetheless, the debate is still open, as a special issue of *Functional Ecology* dedicated to metabolic scaling presents, in the same volume, one study showing evidence against universal metabolic allometry (Bokma, 2004), and another arguing in favour of the predominance of quarter-power scaling (Savage et al., 2004). Interestingly, another more recent study re-evaluated BMR scaling in birds, restricting their comparison to studies that met criteria for BMR, and they showed an exponent of 2/3 for 126 species of birds (McKechnie and Wolf, 2004).

In ectotherms, Clarke and Johnston (1999) compiled resting metabolic rate data from 138 studies, including 69 species of fish. The overall interspecific scaling exponent was 0.80, and the mean intraspecific exponent was 0.79. A normal distribution of intraspecific exponents varying between 0.40 and 1.29 was observed. The distribution frequencies of the exponent intervals 0.55-0.65, 0.65-0.75, and 0.75-0.85 were about 21, 34 and 29% respectively. Similarly, Bokma (2004), compiled data for 113 fish species, and showed that the exponent
values ranged between 0.5 and 1 among fish species, and that neither 2/3 nor 3/4 were supported and concluded that there is no single universal scaling power.

All of these studies tend to indicate that there is no universal scaling coefficient for BMR, or for metabolic rates in other situations (such as maximal aerobic exercise), although the debate remains open (Bokma, 2004, Savage et al. 2004). Nonetheless, if there is no universal value for $b$, then searching for a single unifying explanation for a 'fictional' 3/4 or 2/3 $b$ value is no longer appropriate. There clearly are factors that drive scaling of metabolic rate, but they do not necessarily impose a 3/4 (or any other) exponent.

**Single site versus multiple sites of control.**

Most work on metabolic scaling has searched for the single driving force or single rate limiting step that sets the scaling exponent. Recent focus has been on material supply, i.e., $O_2$ and/or substrates as the rate-limiting process (West et al., 2000; Banavar et al., 1999). Unfortunately, our mechanistic understanding of metabolism has not yet identified such a master controller of organism metabolism, instead growing evidences show that control is shared among multiple steps. Physiologists have developed a more integrative view of metabolism, one that considers control to be distributed across multiple sites and may involve processes involved in both supply and demand. At the biochemical level, a set of formalised theories, termed metabolic control theory, was developed to sort out the contribution of various steps in a metabolic pathway to overall pathway flux (Fell, 1997). This theory was developed largely to account for flux through biochemical pathways, but there is no conceptual barrier to its application to physiological processes such as whole animal metabolism. Indeed, several investigators have applied metabolic control analysis to energy metabolism (Fell and Thomas,

To sort out the contributions of each step in a pathway to the control of net metabolic flux, for example net O$_2$ flux, experimenters determine the fractional change in organismal O$_2$ flux caused by a fractional change in flux capacity through any given step or process in the path of O$_2$ from lungs to mitochondria in working tissues. The fractional change in organismal flux divided by the fractional change in capacity represents the control coefficient at each step. To illustrate, if the O$_2$ flux capacity of the lung is increased by 50% but only a 25% change in overall O$_2$ flux is achieved, the control coefficient for the lung is 0.5, equal to the fractional change in overall O$_2$ flux / fractional change in lung flux capacity. According to the summation theorem (Fell, 1997), the control coefficients in the pathway add up to one. In a system with a classical single rate-limiting step, the control coefficient for that step would be essentially 1; i.e. all control is vested in this process. The latter situation is never found in metabolic systems of any complexity (Fell and Thomas, 1995; Jones, 1998). Hence the usually explicit, sometimes implicit, assumption of classical allometry studies (West et al., 2000; Banavar et al., 1999; Bejan, 2001), that there is a single rate-limiting step or process that accounts for the $b$ value in equation 1, does not appear to apply to living systems. Realising the roles of multi-site contributions to control of metabolism permits us to take a completely different approach to accounting for observed $b$ values.

Scaling of physiological processes

The study of physiological and metabolic processes scaling has been a fruitful area of research. Some early work focused on the metabolic consequences of the scaling of organ mass. In humans, about 75% of BMR is accounted for by metabolically active organs (liver, brain,
kidney, heart, lungs), which account for about 8% of total body mass (Aschoff et al., 1971).
The scaling of organ size has been repeatedly investigated and the exponent was shown to be
organ-specific ranging from 0.70 for the brain and 1.0 for the heart (Stahl, 1965). Moreover,
organ mass-specific metabolic rate was also shown to be organ-specific, whether tissue slices or
isolated cells were used to estimate organ mass-specific respiration rate (Kleiber, 1941; Krebs,
1950; Couture and Hulbert, 1995; Porter, 2001). Thus, combining organ size and mass-specific
metabolic rate scaling should yield organ-specific total metabolic rate scaling, as shown by the
study of Wang et al. (2001) where the exponents ranged from 0.60 for liver and 0.86 for the
heart metabolic rate. Thus, no universal quarter-power is found at the organ level.

The scaling of physiological and biochemical processes has been studied in the context
of basal metabolic rate. The work from Hulbert, Else and colleagues (Hulbert and Else, 2000)
explored the cellular bases of maintenance costs, mainly focusing on processes associated with
biological membranes. These authors showed that organ and cell metabolic rate scaling can be
understood by many membrane associated processes, such as maintenance of ion and proton
gradient across cell and mitochondrial membranes. The activity of the Na$^+$ pump has been
shown to scale with body mass, and is apparently organ-specific (Couture and Hulbert, 1995).
Moreover, proton leak across mitochondrial membranes was also shown to scale with body
mass (Porter et al., 1996) and is apparently taxon-specific (Else et al., 2004). The composition
(polyunsaturation level) of cell and mitochondrial membrane also scales with body mass; this
has led to the concept of membranes as pacemaker of metabolism (Hulbert and Else, 1999).

The research program of Weibel, Taylor and colleagues (Weibel, 2000), has been
fruitful in providing physiological correlates of $\dot{V}O_2$ max in mammals. Interestingly, these
authors showed that the many systems involved in providing locomotory muscles with $O_2$ and
nutrients during maximum aerobic exercise, appear to scale with similar exponent values,
around 0.85. Flux capacities at the biochemical levels have also been investigated in mammalian skeletal muscle, where the mitochondrial enzyme citrate synthase activity was shown to scale to the 0.9 exponent (Emmett and Hochachka, 1981). Further examination of skeletal muscle biochemistry also showed that for 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), the biochemical machinery involved in anaerobic energy production scaled positively. This has been described as a violation of the classic metabolism and body size scaling paradigm (Somero and Childress, 1980).

Multiple contributors, multiple controllers as causes of the allometric scaling exponent

We propose to use a framework where multiple contributors account for whole-animal metabolic rate scaling. Using this framework, the multiple-causes model of allometry, there are multiple contributors to control, MRᵢ, each with its own characteristic bᵢ value, that along with their control contributions in sum determine the value of the b scaling coefficient for overall energy metabolism (Figure 4.1). The concept of an allometric cascade arises from the layering of function at various levels of organisation. The numerous steps involved in intracellular pathways of ATP demand and supply, each with its own characteristic scaling exponent (bᵢ) and control coefficient (cᵢ) values, are at the base of the cascade. Other key steps, such as transporters, exchangers, and pumps, function at the membrane-based cell surface, interfacing and integrating intra- and extra-cellular functions. Not all cells are identical of course, so another layering of function is found at organ and tissue levels of organisation, where organ physiologies are integrated into organism MR. Thus the overall scaling of MR is a consequence of the interaction of scaling of these various “functional units” involved in specific metabolic states. In short, we view this multi-cause model of scaling as an allometric cascade, with the b value for overall energy metabolism being determined by the control coefficients (cᵢ) and the bᵢ
values for all major steps in the biochemical and physiological pathways of energy demand and energy supply that in sum make up whole-body MR (see Fig. 4.1).

To express this concept, we used a simplified view where processes are linked in parallel, although metabolism consists of a mixture of processes in parallel operating in concert with pathways in series. Nonetheless, this simplified view allows the expression of the relationship as

\[ MR_{\text{tot}} = MR_1 + MR_2 + MR_3 + \ldots + MR_i \]  (2)

\[ MR_i = c_i M_b^{b_i} \]  (3)

\[ MR_{\text{tot}} = \sum MR_i \approx \sum c_i M_b^{b_i} \]  (4)

According to this framework, total metabolic rate in a given metabolic situation, \( MR_{\text{tot}} \), is the sum of its contributors, \( MR_i \) (Eq. (2)). In turn, each contributor can have its own scaling exponent, \( b_i \), and contribute to total metabolic rate proportional to its percentage contribution, \( c_i \) (Eq. (3)). Total metabolic rate can thus be approximated by Eq. (4), where \( MR_{\text{tot}} \) is approximately equal to the sum of its contributors following their own power functions.

The objective is to test whether this allometric cascade model can account for the observed empirical data. To test if power relationships result from multiple contributors, we first performed simulations using 6 hypothetical contributors that scale with exponents ranging from 0.5 to 1.0. Variable values of \( c_i \) were used to test the impact on the scaling relationship obtained from such a summation. Another approach, used to support the validity of multiple contributors to metabolism, was to compile data from the mammalian literature to obtain \( b_i \) and \( c_i \) values of the various processes involved during maximal metabolic rate (MMR). This metabolic situation was evaluated because under these conditions, physiological systems are
likely to approach maximal capacity and be limited by supply. Finally, the mammalian literature was also surveyed to identify the $b_i$ and $c_i$ values of major processes involved during basal metabolic rate (BMR), because most studies of metabolic rate scaling have examined BMR.

**Methods**

*Simulations*

To test the validity of the allometric cascade framework, we first performed simulations of data based on hypothetical scenarios. Simulations were performed over a 10 order of magnitude arbitrary body mass range (0.0001 to 1000000). The summation of 6 processes with power functions with $b_i$ exponents ranging from 0.5 to 1 was performed for each body mass log unit, which yielded the summed data points fitted with a power function. Variable $c_i$ values were used to test the effect on $b$ values of the fitted function. Finally, residuals of the whole-animal BMR scaling curve reconstructed from organ metabolic rates by Wang et al. (2001) were compared with residuals obtained from empirical data on whole-animal BMR scaling (Savage et al. 2004).

*Mammalian MMR.*

To further test the application of the allometric cascade, we examined whether this model was consistent with values for MMR obtained or estimated from the mammalian literature. Because rigorous estimates of control coefficients for most contributors we identified were not available, we approximated $c_i$ values from available literature to identify a range of coefficient for the various processes. This analysis was performed using the body mass range usually covered by mammalian studies, from 0.01 to 1000 kg. To apply the model, the
contributors to MMR and their scaling relationship were gathered from the literature. On the energy supply side, the $c_i$ values are estimated from the data of Wagner (1993) for human $\dot{V}O_2\text{max}$ performance. In these analyses, control contributions from cell metabolism were ignored. However, we assume that mitochondrial metabolism displays a $c_i$ value well above zero (for example, training increases mitochondrial volume by $\sim 25\%$ which correlates with a $\sim 5\%$ increase in $\dot{V}O_2\text{max}$ (Hochachka, 1994), indicating an approximate $c_i$ value of $\sim 0.2$). To accommodate the mitochondrial data and to have all the $c_i$ estimates add up to 1.0 in exercising humans, the estimates from Wagner (1993) must be multiplied by 1/1.2 or 0.8. Thus for alveolar ventilation $b_l = 0.80$ (Stahl, 1967) and $c_i = 0.12$ (0.16 from Wagner’s data x 0.8). For pulmonary diffusion, $b_l = 1.08$ (Weibel, 2000) and $c_i = 0.20$ (Wagner, 1993). For cardiac output (Bishop, 1999), $b_l = 0.879$ while $c_i = -0.32$ (Wagner, 1993). Capillary volume densities scale with $b_l = 0.89$ (Weibel, 2000), with $c_i = 0.20$ (Wagner, 1993) for $O_2$ diffusion from capillaries to mitochondria. Mitochondrial volume densities and hence their maximum oxygen consumption rates scale with $b_l = 0.87$ (Weibel, 2000) while $c_i = -0.16$ (Hochachka, 1994; Hochachka and Somero, 2002).

On the energy demand side, under MMR conditions, over 90% of $O_2$ consumption (Hochachka and Somero, 2002; Weibel, 2000) is directed towards ATP synthesis for muscle work, i.e., for actomyosin (AM) ATPase and the $Ca^{2+}$ pump. What we need to complete our calculations are $b_l$ and $c_i$ values for both of these major ATP sinks. For AM ATPase, $b_l = 0.77$ (Lindstedt et al., 1985), and at low-to-moderate exercise intensities it contributes significantly to control of overall metabolic rates (Hochachka et al., 1991; Korzeniewski, 2000). Allometric data on the $Ca^{2+}$ pump also are not abundant, but since muscle contraction frequency is directly correlated with this activity, an in vivo estimate of $b_l = 0.86$ is provided by scaling of stride frequency in a mouse to horse range of body sizes (Heglund et al., 1974). Similar scaling
coefficients are obtained for the Ca\textsuperscript{2+} pump catalytic capacities in hearts of small and large animals (Hamilton and Iannuzzo, 1991). Estimates of \( c_i \) values for AM and Ca\textsuperscript{2+} ATPase can be obtained by comparing the impact of change in their capacities upon tissue respiration. AM concentrations and activities are 3-4 fold higher in FOG (fast twitch oxidative glycolytic) fibres than in slow fibres, while Ca\textsuperscript{2+} pump densities are about 1.7 fold higher (Szentesi et al., 2001); together these can ‘drive’ O\textsubscript{2} delivery about 2.4 times faster (Armstrong and Laughlin, 1985; Hochachka et al., 1991) (linear over a broad range of submaximal work rates). AM accounts for 1.92 fold activation, while Ca\textsuperscript{2+} cycling accounts for the rest (~48% of the activation). Thus under these conditions, the \( c_i \) value (change in overall metabolic flux achievable/change in actomyosin catalytic capacity) equals 1.92/3.0 or 0.63, while for the Ca\textsuperscript{2+} pump the \( c_i \) equals ~0.48/1.7 or ~0.28. Since these values are calculated with respect to O\textsubscript{2} flux, in total the two processes contribute up to 91% of the control under submaximal work. However, since \( c_i \) values may approach zero at MMR (Jeneson et al., 2000), in our simulations, we used two \( c_i \) values for actomyosin and Ca\textsuperscript{2+} ATPases, 0.63 vs. 0 and 0.28 vs. 0, respectively. A summary of these values is given in Figure 4.4A and Table 4.1.

**Mammalian BMR**

The concept of allometric cascade was also tested against empirical data available for BMR. On the energy supply side, under BMR conditions, all the oxygen delivery steps display a large excess capacity (Weibel, 1984; Hochachka and Somero, 2002). Thus, small \( c_i \) values were selected for each of these steps. The \( b_i \) and \( c_i \) values chosen for alveolar ventilation (Stahl, 1967) are \( b_i = 0.80 \) and \( c_i = 0.01 \). For pulmonary diffusion (Weibel, 2000), \( b_i = 1.08 \) and \( c_i = 0.01 \). For cardiac output (Bishop, 1997) \( b_i = 0.76 \) and \( c_i = 0.01 \). For diffusion from capillaries to mitochondria, \( b_i = 0.89 \) (as above) while \( c_i = 0.01 \). For mitochondrial O\textsubscript{2} consumption, we were confronted with the issue of what state the mitochondria operate in under BMR conditions. As
indicated above, state 3 mitochondrial respiration (saturated with all required substrates) is expected to scale to the 0.87 power. However Porter et al. (1996) found a positive scaling of the respiratory control ratio, RCR, with a $b_t$ value of 0.121, indicating that state 4 respiration scales with a lower exponent than state 3. It is likely that under BMR conditions mitochondria function at a small fraction of their state 3 rates (approaching state 4), thus we considered that $b_t$ values of 0.87-0.12 = 0.75 and $c_t$ values of about 0.01 would be reasonable estimates for mitochondrial respiration under BMR conditions.

On the energy demand side of the ATP turnover cycle, under BMR conditions the two major energy sinks are the Na$^+$ K$^+$ ATPase and protein turnover (Rolfe and Brown, 1997; Hulbert and Else, 2000). For Na$^+$ K$^+$ ATPase, Couture and Hulbert (1995) determined an in vivo $b_t = 0.72$ and in our simulation the $c_t$ was chosen to vary from 0.70 to 0.35. Estimates of $c_t$ values for Na$^+$ K$^+$ ATPase can be obtained by comparing the impact of change in Na$^+$ K$^+$ ATPase capacity upon tissue respiration. In vertebrate liver, a 5.6 fold change in Na$^+$ K$^+$ ATPase capacity ‘drives’ a 1.63 fold change in $\dot{V}O_2$, while in vertebrate brain, a 3.78 fold change in Na$^+$ K$^+$ ATPase capacity ‘drives’ a 1.63 change in $\dot{V}O_2$; thus the ratios of change in metabolic flux capacities/change in Na$^+$ K$^+$ ATPase capacities, the $c_t$ values, are 0.77 (for liver) and 0.43 (for brain). We do not have global $c_t$ values for whole body rates, but since Na$^+$ K$^+$ ATPase activities account for a large % of BMR of numerous animals (Rolfe and Brown, 1997; Hulbert and Else, 2000), the $c_t$ values estimated for the liver and brain are probably fairly representative. In some tissues, such as kidneys, the values may be higher; in some, such as non-working muscles, they may be lower (Hulbert and Else, 2000). That is why in our simulation (Figure 4.4B), two widely differing $c_t$ values, 0.70 and 0.35, are used to illustrate the effect on overall scaling behaviour.
A second major energy demand process under BMR conditions is protein synthesis.
Previous allometric studies of protein synthesis rates establish $b_i = 0.77$ (Waterlow, 1984;
Brody, 1945), but experimental studies allowing quantification of $c_i$ coefficients for this process
are not abundant. Nevertheless, in vitro studies (Fuery et al., 1998) provide a $c_i$ value of 0.11
for protein synthesis in liver slices of amphibians. In humans and rats, whole-organism studies
of protein synthesis and metabolism usually find $c_i$ values that are somewhat higher (Rolfe and
Brown, 1997; Hochachka and Somero, 2002). For our simulation, with the Na$^+$ K$^+$ ATPase $c_i$
set at 0.70, we selected for protein synthesis a value of $c_i = 0.10$. To complete our simulation,
we also included data for three more ATP demand processes: Ca$^{2+}$ ATPase plus urea and
glucose biosyntheses. As above, a value of $b_i = 0.86$ (Heglund et al., 1974) is our best current
estimate for the Ca$^{2+}$ pump, while because of a low contribution to BMR we assume a relatively
low value of $c_i = 0.05$. For urea synthesis (Brody, 1945) $b_i = 0.77$ and we selected $c_i = 0.05$.
Similarly, for gluconeogenesis (Weber et al., 1997), $b_i = 0.76$ and we selected $c_i = 0.05$; the
latter two $c_i$ values are again similar to % contributions of these processes to BMR (Rolfe and
Brown, 1997). We consider these low estimates to be reasonable, since so far no studies have
found large contributions of ureagenesis or glucogenesis to control of basal metabolism
(Hochachka and Somero, 2002; Rolfe and Brown, 1997). With the Na$^+$ K$^+$ ATPase $c_i$ value set
at 0.7, the values for all the ATP demand processes then are 0.10, 0.05, 0.05, and 0.05
respectively; these double when the Na$^+$ K$^+$ ATPase $c_i$ value is reduced to 0.35. The two $c_i$
values for protein synthesis bracket the % contribution (Rolfe and Brown, 1997) of this ATP
demand process to BMR. A summary of these values is given in Figure 4.4B and Table 4.1.
Results

Simulations

The simulations considered 6 contributors with scaling exponents ranging from 0.5 to 1.0. The resulting data points could be fitted with a power function with a coefficient of determination greater than 0.99 (Fig. 4.2). The coefficient of determination increased when smaller size ranges or narrower exponent ranges were used (e.g. 0.65-0.85) (data not shown). The data points obtained from summed MR appear curvilinear. Using the data from Wang et al. (2001), the residual variation from reconstructed MR was analysed and plotted against body mass. The distribution of residuals was compared with the distribution obtained from empirical data (Savage et al., 2004). Figure 4.3 shows that the distribution of residuals obtained from empirical data appears to be more variable at both ends of the body mass range, which agrees with the distribution obtained from reconstructed metabolic rate.

Mammalian MMR

To reconstruct MMR metabolic rate, two different scenarios were tested. The first set of $c_i$ values that were used (Table 4.1 and Fig. 4.4A), where the control was shared among steps on the supply side, yielded a scaling exponent of 0.92 ($r^2 > 0.999$). When processes involved in ATP demand (AM ATPase and Ca$^{2+}$ pump) were used, the $b$ value obtained was 0.82 ($r^2 > 0.999$).

Mammalian BMR

The analyses for BMR also contrasted two different situations. In this case, the processes involved in energy demand dominate, and were also varied in proportion. When protein synthesis and Na$^+$ pump control was almost equally shared ($c_i$ values in Table 4.1 and
Fig. 4.4B), the reconstructed exponent was 0.79 ($r^2 > 0.999$). When $c_i$ value for Na$^+$ pump was set to 0.70, the $b$ value decrease to 0.76 ($r^2 > 0.999$).

Discussion

*Whole-animal metabolic rate as the sum of its parts*

The main objective of the simulation performed was to demonstrate that whole-animal metabolic rate scaling can be explained by multiple contributors. By using a simplified model of multiple-contributors to metabolic rate, we showed that whole-animal energy expenditure can be reconstructed by the summation of the various contributors involved. This simple empirical summation of various contributors has been subject to criticism (West et al., 2003; Banavar et al., 2003) based in the fact that the equation used in our original contribution (Darveau et al., 2002) was flawed. The origin of this dispute resides in the fact that the original equation used the equal sign, implying mathematical summation. Instead, equation (2) shows that metabolic rate is the sum of its contributors, which are described by power functions in equation (3), thus metabolic rate is approximately equal to equation (4) as power functions cannot be mathematically summed, but empirical summation yielded a relationship indistinguishable from a power function (Fig. 4.2). Moreover, the imperfection of the obtained power function was analysed by comparing the residuals obtained from empirical BMR measurements (Savage et al., 2004), and the residuals of the reconstructed BMR performed by Wang et al. (2001). The curvilinear relationship found for the residuals of the reconstructed power function are similar to residuals obtained from the empirical relationship (Fig. 4.3), which suggests that multiple contributors can account for the empirical power function describing metabolic rate scaling. In addition, many workers have stressed the fact that metabolic rate scaling relationships are not necessarily perfect power functions, as residual
variation is greater at both ends of the body mass distribution (Lovegrove, 2000) and that possibly small and large mammals would follow different power functions, close to 2/3 in small mammals, and close to 1.0 for large mammals (Dodds et al., 2001; Makarieva et al., 2003). Finally, Schmidt-Nielsen (1984) wisely recommended that biologists remember that allometric equations are mathematical descriptions of empirical data, and that they are not biological laws!

We applied this approach to the physiological processes that appeared dominant in contributing to ATP turnover rate, but various levels of organisation can be investigated using such analysis. Interestingly, while developing this concept, Wang et al. (2001), used the same approach to reconstruct Kleiber's curve from data available for organ metabolic rate. Wang et al., (2001) compiled data for organ size and mass-specific respiration rate scaling for mammals. Their results are summarised in Figure 4.5, and the summed whole-animal metabolic rate yielded the equation, $303 M_b^{0.76} (r^2 = 0.999)$, which was impressively close to Kleiber's original observation, $307 M_b^{0.74}$ (Kleiber, 1932). This example illustrates that whole-animal metabolic rate scaling can not only be explained by one single driving force enforcing its universal exponent, but that multiple-contributors can as easily accommodate empirical observation of this phenomenon. Such approach has also been applied intraspecifically in fish and whole-animal metabolic rate scaling could also be reconstructed by the summated tissue respiration (Oikawa and Itazawa, 2003).

Scaling of maximum metabolic rate

Contrasting scaling relationships in different metabolic situations permits us to test the universality of available scaling models. For example, single-cause model of allometry based on distribution networks (West et al., 2000; Banavar et al., 1999) would predict that the universal 3/4 scaling exponent applies to MMR. Using multiple-cause, one realises that most
contributors to MMR scale with exponents higher than 3/4 (Table 4.1), which would yield maximum metabolic rate scaling exponents between 0.82 and 0.92 (Fig. 4.4A). Empirical measurements of MMR and its contributors has been the core of Weibel and colleagues research program (Weibel, 2000), and the most recent analysis of MMR data shows exponent values around 0.87 (Weibel et al., 2004), consistent with the predictions of our model. Furthermore, these authors also demonstrate that the aerobic capacity of locomotory muscles is tightly correlated with MMR scaling, as capillary volume and mitochondrial density scale with body mass with nearly identical scaling exponents (Weibel, 2000; Weibel et al., 2004) as that observe for MMR (Table 4.1). In light of these empirical measures, it appears that the scaling of distribution networks do not obey the 3/4 “law”, but instead higher exponent values are observed. A similar conclusion was reached by Bishop (1999) who studied MMR and cardiovascular scaling in birds and mammals. Moreover, the study of Guttierez (2002), showed that the integration of fractal networks and organ volume yields a mixture of scaling exponents; thus, no universal quarter-power “law” would be predicted for each organ. Finally, other studies have tested the assumption of fractal networks for lungs and circulation, and it was concluded that lung dynamic does not behave according to a fractal network (Sapoval et al., 2002), and that vascular system modeling does not necessarily use fractal geometry assumptions (Kurz, 2000; Godde and Kurz, 2001). Thus it appears that the assumptions of fractal networks are questioned for the lung and circulation systems, moreover, the predictions of distribution networks theories do not agree with empirical observations for MMR scaling, which is the metabolic situation where the limitations of the delivery system would be the most important.

The main contrast between our approach and recent single-cause models of allometry, is that we also incorporate processes involved in energy demand in our understanding of scaling. Although during MMR delivery systems are likely to be the main controllers of metabolism
(Weibel, 1984), the functional relationships and correlated evolution between physiological processes involved in supply and demand remains to be fully understood. The recent study by Weibel et al. (2004), showed that athletic species have a greater aerobic scope, and their MMR scaling exponent reached 0.94. In the present analysis, the scaling exponents of the two major ATP consuming processes involve during MMR have been estimated, that is actomyosin ATPase and Ca^{2+} pump (Table 4.1). Interestingly, metabolic control analysis has been applied to contractile muscle cells, and actomyosin ATPase was shown to contribute significantly to overall metabolic rate during low to moderate exercise (Korzeniewski, 2000). Thus, when most of the control was applied on the ATP demand side, we predicted an exponent of 0.82 (Fig. 4.4A). I therefore propose that metabolic rate scaling studies should integrate the functional relationships and correlated evolution between supply and demand to understand MMR scaling patterns. The research presented in Chapter 2 and 3 illustrates this point, as hovering flight metabolic rate scaling and evolution could be understood via insect flight biomechanics, and flight metabolic rate was shown to be correlated with biochemical processes involved in energy supply. It is therefore possible to explain metabolic rate scaling without involving the single limitation of distribution networks, as much is known about the scaling of form and function associated with locomotion.

According to single-cause models of allometry explaining a 3/4 power "law", the above discussion on MMR scaling cannot be explained by such model as it deviates from the predicted exponent value. Moreover, the predictions from single-cause models imply that there is no relationship between MMR and BMR, as different scaling patterns are observed. However, many researchers have established links between MMR and BMR and their scaling. Studies performed on mammals and birds showed the correlated evolution between mass-corrected active and resting metabolic rate (Ricklefs et al., 1996; Rezende et al., 2002). The
relationship between active and resting metabolic rate has also been suggested in insects (Reinhold, 1999). Such connections between activity level and cost of maintenance have also been the basis for the activity capacity hypothesis for the evolution of endothermy (Bennett and Ruben, 1979), and more recent analysis of determinants of metabolic scope (Kroshnyunas and Gerstner, 2003). Further work connecting MMR and BMR would be of great interest and will provide insights into metabolic rate evolution and its scaling in various states.

**Scaling of basal metabolic rate**

Less is known about the control of BMR than of MMR. To identify a starting point, we need to review what processes add up to the whole body metabolism we refer to as BMR. In the laboratory rat at rest, ~70% of the whole body O_2 consumption is used by the mitochondria to produce ATP for several ATP sinks, including protein turnover, Na^+ pump, Ca^{++} pump, actomyosin ATPases, gluconeogenesis, ureagenesis, mRNA synthesis, and substrate cycling, contributing approximately 20-25, 20-25, 5, 5, 7, 2.5, <2, and <2% respectively (Rolfe and Brown, 1997) to BMR. The relative contributions to MR of Ca^{++} and actomyosin ATPases of course rise as the rate of muscle work increases (discussed above).

To apply the multiple-cause model of allometry, we first asked what energy demanding processes dominate BMR, and what scaling relationship is known for each process. The cost of maintenance appears to be dominated by the maintenance of membrane potential achieved by Na^+ pump activity and protein turnover rate. Using these two energy demanding processes that dominate BMR energy turnover rate, as well as Ca^{2+} pump, glucose and urea synthesis led to estimated scaling exponents for BMR of between 0.76 and 0.79 (Fig. 4.4B). It thus appears that BMR scaling can be determined by the scaling of its contributors, which scale close to the 3/4 exponent in "classic" scaling studies (Table 4.1). Further studies of Na^+ pump and protein
turnover scaling would be particularly interesting to perform on birds, as recent evidence showed that their BMR appear to scale to the 2/3 power (McKechnie and Wolf, 2004), and that some physiological processes appear to scale differently in birds and mammals (Else et al., 2004). Finally, the choice of species used in scaling studies would probably affect the exponent values of both whole-animal and physiological levels, as shown in birds (McKechnie and Wolf, 2004) and mammals (White and Seymour, 2004).

Recent single-cause models trying to explain BMR 3/4 scaling exponent are based on the optimal design of distribution networks and the limitation imposed by this optimal design. For example, West et al. (2002) argue that cell cultures derived from animals over a wide range in body mass should eventually all have similar metabolic rate, as cells originally from large mammals would no longer be constrained by a fractal delivery system. The opposite argument can also explain such observation, as the cells are no longer tuned by metabolic demand associated with size, such that a muscle cell will no longer contract at frequency set by animal size, a liver cell will no longer respond to whole body glucose cycling and so on. Interestingly, when cellular respiration rate is measured in cells isolated from animal tissue, the scaling relationship applies, suggesting that cellular machinery scales as shown repeatedly by several research group (Porter and Brand, 1993; Couture and Hulbert, 1995; Porter et al., 1996). Therefore, the demonstration of West et al. (2002) using various cell types and even cancerous cell lines, tells us more about cellular differentiation in vitro, than metabolic rate scaling.

At BMR, it seems clear that demand processes rather than supply must set MR, since supply processes are sufficient to support MMR. For example, hibernators during normal situations exhibit basal metabolic rate scaling, however, when cost of thermoregulation is reduced during hibernation, mass-specific metabolic rate becomes independent of animal's size (Singer et al., 1995). It is hard to deny the effect of body size on temperature maintenance in
endotherms, but the multiple-contributor framework of allometry, also implies that the cost of maintenance of any organism will be size dependent. The above discussion of MMR scaling showed the effect of body mass on locomotory machinery, which could greatly contribute to the scaling of protein turnover and maintenance cost associated with locomotion and size. The contribution of demand processes to BMR scaling has yet to be fully resolved, but in our view, should remain central to the BMR scaling discussion.

_intrinsic and extrinsic factors affecting metabolic and physiological scaling_

The basic physiological processes we have identified as contributors to metabolism are affected by many intrinsic and extrinsic factors. The study of Wang et al (2001) and Else and Hulbert (1985), illustrates that the level of organisation can be viewed as an intrinsic factor, such as organ size and mitochondrial content. These studies confirm differential scaling of various organs and their mitochondrial membrane content, which in our view, undoubtedly contributes to the scaling of MR. Thus, comparison of species groups that would differ in their organ size proportion and metabolic rate would be reflected in their whole-animal metabolic rate scaling. Intrinsic factors are found at all levels of organisation. Variation in membrane composition is another intrinsic factor that may influence scaling coefficients. In mammals at rest, 10% of the oxygen consumed by the animal is non-mitochondrial, while another 20% of the O₂ consumed is used to maintain mitochondrial membrane potential affected by the leak of protons (Rolfe and Brown, 1997). Allometric scaling of proton leak across the mitochondrial inner-membrane displays a $b_l$ exponent of 0.86 in mammals (Porter and Brand, 1993; Porter, 2001). Proton leakiness can be influenced by membrane composition (Porter et al., 1996), and polyunsaturation levels of biological membranes decrease (Hulbert and Else, 1999, 2000) as body size increases. This however, appears to be taxon specific, as recently shown by Else et al. (2004), where birds display different scaling patterns. Although more work is required, it is
already evident that membrane composition provides another example of intrinsic effects on MR. The scaling of the mitochondrial proton leak and Na$^+$ pump activity of cell membranes is in part a function of the leakiness of membranes in which it operates, which is a consequence of membrane composition.

Field biologists frequently emphasise that factors such as temperature, pressure, nutritional preferences, water availability, and so forth may be ‘ultimate causes’ or ‘ultimate explanations’ for why specific groups of organisms show specific allometric behaviour. Appeal, for example, is made to this level of explanation for different scaling exponents for field metabolic rates (FMR) when comparing reptiles, birds, and mammals (Nagy et al., 1999). Nagy and Bradshaw (2000) also found lower exponents for field metabolic rate of Australian marsupials compared to placentals and lower intercepts for desert forms, with additional effects of nutrient source (especially noted in studies of carnivorous, insectivorous, or nectarivorous birds). Similar explanations apply to the phylogenetically or environmentally linked scaling patterns noted by Lovegrove (2000). At a finer scale, Mueller and Diamond (2001) showed that BMR, food intake, intestinal glucose uptake capacities, and presumably scaling patterns are related to the net primary productivity of the habitat of origin of five mice species. We interpret all such forces (in addition to purely abiotic ones such as temperature, pressure, or osmotic pressure) as extrinsic factors that effect scaling relations of physiological processes. So called ‘ultimate causes’ thus are not ‘causes’ in a mechanistic sense, but only in the sense that they act through ‘proximal causes’ or ‘proximal mechanisms’ such as outlined in our model.

Conclusions

In summary, most earlier analyses of the scaling of metabolic and physiological processes have looked for a single process that scales with a universal $b$ exponent; their models
then assumed that this single process enforces the scaling behaviour of all other biochemical and physiological rate functions. We here refer to all such interpretations as single-cause models of scaling. The only way these could be valid is if the selected single process behaved as a master rate-determining step in metabolism and physiology, a concept that was questioned in metabolic regulation research some 4 decades ago and has since been virtually discarded. Taken literally, if a single process, such as O₂ or fuel delivery, is rate limiting for BMR, there can be no room left for scope for activity. Instead, at BMR, delivery systems possess large excess capacity and it appears that whole-animal MR is determined by energy expenditure rather than control by supply. In addition, if such single cause would explain metabolic rate scaling, it should apply to MMR and possibly be reflected in BMR, but instead these two states scale differently which is incompatible with single 3/4 power “law”. For these reasons and many others (Hochachka and Somero, 2002), the concept of a master rate limiting step was replaced by concepts of multi-site controls, with many processes contributing in varying degree to regulation of overall whole-organism metabolic and physiological rates (Thomas and Fell, 1998; Jones, 1998; Jeneson et al., 2000; Hochachka and Somero, 2002). When applied to the problem of scaling, the multi-site concept of allometry states that the product of two parameters (b, the scaling exponent, and c, the control coefficient in equation (4)) for all major control sites in ATP turnover pathways determine the overall scaling behaviour of whole-organism bioenergetics.

Finally, the proposed multiple-cause framework of allometric scaling can be applied in various ways. The approach used in the present study represents a bottom up approach, where whole-animal metabolic rate scaling can be reconstructed by the sum of its contributors. Such approach, however, is limited to species group largely studied in order to compile the necessary information (b and c values) for the major physiological processes involved. Other approaches
can be used to test the validity of this framework. For example, studies have been applied to humans and showed the differential scaling in different metabolic state (Batterham and Jackson, 2003). In addition, studies have tested if various component of the allometric cascade indeed scale with exponents different than the 3/4 (Weibel et al., 2004; Else et al., 2004). Finally, a top down approach could be used to test the framework. Such approach has been used in Chapters 2 and 3, where the scaling of whole-animal metabolic rate has been correlated to the evolution of physiological processes involved in its control.
Table 4.1. Values estimated for $b_i$ and $c_i$ for various steps in ATP supply and demand pathways in mammals under aerobic MMR and under BMR conditions. For MMR conditions, the functions listed in order are alveolar ventilation ($V_A$), pulmonary diffusion ($D_{lung}$), cardiac output ($Q$), capillary-mitochondria diffusion ($D_{tissue}$), cytosolic and mitochondrial metabolism ($M_{mito}$) plus actomyosin ATPase (AM ATPase) and the Ca$^{2+}$ pump. The same abbreviations are used for BMR conditions, in addition to the Na$^+$ pump and protein, urea and glucose synthesis. See methods for literature sources.

<table>
<thead>
<tr>
<th>Process</th>
<th>$b_i$</th>
<th>$c_i$ value to estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$b_{max}$</td>
<td>$b_{min}$</td>
</tr>
<tr>
<td><strong>MMR conditions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_A$</td>
<td>0.80</td>
<td>0.12 0.01</td>
</tr>
<tr>
<td>$D_{lung}$</td>
<td>1.08</td>
<td>0.20 0.02</td>
</tr>
<tr>
<td>$Q$</td>
<td>0.88</td>
<td>0.32 0.03</td>
</tr>
<tr>
<td>$D_{tissue}$</td>
<td>0.89</td>
<td>0.16 0.01</td>
</tr>
<tr>
<td>$M_{mito}$</td>
<td>0.87</td>
<td>0.20 0.02</td>
</tr>
<tr>
<td>AM ATPase</td>
<td>0.77</td>
<td>0.00 0.63</td>
</tr>
<tr>
<td>Ca$^{2+}$ pump</td>
<td>0.86</td>
<td>0.00 0.28</td>
</tr>
<tr>
<td><strong>BMR conditions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_A$</td>
<td>0.80</td>
<td>0.01 0.01</td>
</tr>
<tr>
<td>$D_{lung}$</td>
<td>1.08</td>
<td>0.01 0.01</td>
</tr>
<tr>
<td>$Q$</td>
<td>0.76</td>
<td>0.01 0.01</td>
</tr>
<tr>
<td>$D_{tissue}$</td>
<td>0.89</td>
<td>0.01 0.01</td>
</tr>
<tr>
<td>$M_{mito}$</td>
<td>0.75</td>
<td>0.01 0.01</td>
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<tr>
<td>Na$^+$ pump</td>
<td>0.72</td>
<td>0.35 0.70</td>
</tr>
<tr>
<td>Ca$^{2+}$ pump</td>
<td>0.86</td>
<td>0.10 0.05</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>0.77</td>
<td>0.30 0.10</td>
</tr>
<tr>
<td>Urea synthesis</td>
<td>0.77</td>
<td>0.10 0.05</td>
</tr>
<tr>
<td>Glucose synthesis</td>
<td>0.76</td>
<td>0.10 0.05</td>
</tr>
</tbody>
</table>
Figure 4.1. Diagrammatic representation of the multi-site model of metabolism allometry. Examples of energy demand (ATP utilizing) processes are shown on the left, each with its own values of scaling exponent, $b_i$, and control coefficient, $c_i$. Similarly, physiological and biochemical examples of energy supply processes (directly or indirectly contributing to ATP synthesis) are shown on the right, again each with its own unique values of $b_i$ and $c_i$. The equation relating these functions to MR (equation 4 in text) is shown. A representative plot of log metabolic rate (MR) vs log body mass ($M_b$) is also shown. Finally, a variety of intrinsic and extrinsic factors are shown to act upon any of the factors in either the energy demand or the energy supply boxes.
$MR_{tot} \approx \Sigma c_i \cdot M^b_i$
Figure 4.2. Relationship between body mass and 6 hypothetical physiological rates scaling (dashed lines) according the power equations shown in the legend. The distribution of $c_i$ values differs between (A) and (B) and affects the sum of contributors (filled diamonds) and $b$ value of the fitted power function (solid line).
Figure 4.3. Relationship between body mass and residuals obtained from the empirical BMR scaling relationship from Savage et al. (2004) (open circles), and from the BMR power function reconstructed from the sum of organ metabolic rate (Fig. 4.5) by Wang et al. (2001) (solid circles).
Figure 4.4. Estimates of scaling exponent, $b$, values for (A) maximum and (B) basal metabolic rates in mammals using the multi-site allometry model. The scaling patterns of the maximum and basal rates (MMR and BMR) were obtained from simulations according to equation (4), using $c_i$ values shown in (A) and (B) and $b_i$ values summarised in Methods section and Table 4.1. The two sets of $c_i$ values yield estimates of maximum ($b_{\text{max}}$) and minimum ($b_{\text{min}}$) values for scaling of MMR and BMR. For simulation purposes, metabolic rate and body mass are given in arbitrary units and the intercept for MMR is assumed to be 10-fold higher than for BMR (arbitrarily set at 1). For reference, slopes of 1.0 and 0.67 are shown.
Figure 4.5. Relationship between body mass and metabolic rate of metabolically active organs (dashed lines) for mammals as compiled by Wang et al. (2001). The summed metabolic rate can be fitted with a power function (solid line) almost identical to Kleiber’s curve, BMR = 307M_b^{0.74} (Kleiber, 1932).
CHAPTER 5
SUMMARY AND CONCLUSIONS

This thesis investigated the evolution of energy metabolism using a lineage of orchid bees that range in body size by over an order of magnitude (50 to 1000 mg). This model system allowed me to use the allometric scaling due to body size as a source of flight metabolic rate variation. I was able to link form and function of flight energetics to the design of metabolic pathways that supply energy. Overall, the research presented throughout this thesis combines evolutionary and mechanistic perspectives in the investigation of energy metabolism evolution. Using the approaches provided by evolutionary physiology (phylogenetically independent contrasts), it is possible to explore evolutionary patterns and mechanisms, but also physiological and biochemical mechanisms. Comparative studies of large-scale physiological patterns, such as metabolic rate scaling, should also combine these different perspectives and balance empirical observations and theories derived to explain the phenomenon.

At the whole-animal level, I demonstrated that variation in body mass was related to a 3-fold range in wingbeat frequency, which was paralleled by mass specific metabolic rate during hovering flight. Variation in wing length and area explained more of the variation in wingbeat frequency than body mass, consistent with the asynchronous flight muscle contraction frequency dependence on the inertial load (i.e. wing mass distribution along the wing length) which affects the resonance properties of the flight apparatus. Moreover, the fine adjustment in wingbeat frequency was determined by the wing loading of the species, as revealed by the analysis of residuals. The wingbeat frequency and wing loading of a species were highly correlated after controlling for both body mass and phylogenetic relatedness. Wingbeat frequency variation could thus be explained by wing size and wing loading. The parallel scaling observed for mass-specific metabolic rate relationship followed the exact same scaling pattern as wingbeat frequency ($b = -0.31$ in both cases), which suggests a functional link of these two
variables. Analysis of body mass and phylogeny-independent data showed the correlated evolution of these two variables. This study thus established links between form and function associated with hovering flight, where the effect of body mass on wing size strongly influenced the wingbeat frequency during hovering flight, which directly impacted mass-specific metabolic rate. The relationship found for body mass and phylogeny-independent wing loading and mass-specific metabolic rate illustrated the link between wing form, kinematics and metabolic rate.

At the biochemical level, I showed that the activity of one enzyme, hexokinase, was proportional to mass-specific metabolic rate. Orchid bee asynchronous flight muscle had enzyme activities similar to that observed for other hovering insects such as honeybees, where high enzymatic flux capacity was observed for glycolysis (HK, PGI, PFK), the Krebs cycle (CS) and the electron transport chain (COX). Among all enzymes measured, only for HK was the scaling relationship similar \((b = -0.33)\) to the scaling of flight mass-specific metabolic rate. Further analysis showed that only HK was correlated with metabolic rate, after controlling for phylogenetic relatedness or body mass. The correlated evolution of HK and flight metabolic rate suggests an important role of this specific reaction on the overall pathway flux rate. The enzyme hexokinase has been identified as a major control site for overall glycolytic flux rate in other highly aerobic tissues (Puijaner et al. 1997; Whitesell et al. 2003; Fueger et al., 2004) suggesting that HK may play an important role in controlling orchid bee flight muscle energy production. The information provided by metabolic control analysis suggests that HK was targeted because of its high degree of control of overall pathway flux. Of the 8 enzymes measured, only HK activity was directly related with interspecific variation in mass-specific metabolic rate or pathway flux rate, which represents a complete hierarchical regulation of this step. Most other enzymes measured were under complete metabolic regulation, where
interspecific variation in flux at those steps was accounted for only by interspecific variation in concentrations of substrates, products or allosteric modulators. This study, combined with the whole-animal level results, suggests that we can link the evolution of energy metabolism from wing morphology to the expression of a single molecule of the flight muscle.

The observations on orchid bees combined with the recent resurgence of interest for metabolic rate scaling led me to explore the effects of body mass on metabolic rate. The recent focus on the $3/4$ power "law" as a universal pattern for metabolic rate relationship with body mass made me re-evaluate the phenomenon. A critical review and analysis of the most recent empirical measurements in mammals, birds and fish clearly demonstrates that a universal $3/4$ power "law" does not explain metabolic rate scaling. In addition, some recent proposals explaining the $3/4$ power scaling are based on the scaling of distribution networks, which is in contrast to my conclusion that, for orchid bees, demand processes may be the most important factors imposing scaling relationships. A modern understanding of metabolism suggests that multiple sites are involved in the control of metabolic rate, and so far no universal controller of metabolism, i.e. single rate-limiting step, such as the rate of material supply by distribution networks, exists. Thus, I proposed a multiple-cause framework to understand the allometric scaling of metabolic rate. This framework can be viewed as an allometric cascade, where the various functions found at the different levels of organisation can be under the influence of body mass, which combined together will add up to whole animal metabolic rate, and its scaling. Using such a framework, I showed that it can predict the scaling exponent value obtained for the scaling of maximum metabolic rate in mammals, which was shown to scale with the 0.87 exponent. I also showed that the lower exponent values observed for basal metabolic rate could be predicted by our model. Thus, the differential scaling found between different animal groups, or in the same group of animals in different metabolic situations,
cannot be explained by single-cause model of allometry, whereas it can be predicted and expected using our multiple-cause framework.

By using a comparative approach, I was able to study energy metabolism from the whole-animal level down to the metabolic pathway. At the whole-animal level, I found strong links between morphology and function associated with locomotion. At the biochemical level, I showed that treating metabolic pathways as a whole, using metabolic control theories as a framework, enables one to identify targets that are correlated with the interspecific variation in pathway flux rate. A research program that integrates multiple evolutionary time scales would be useful to further investigate such functional relationships and evolutionary processes. For example, further studies using hovering insects as a model system would benefit from intraspecific or interpopulation studies that would scrutinise the relationship between wing size, wingbeat frequency, metabolic rate and metabolic pathways design. Such studies would provide insights into fine relationships between the functionally linked variables identified, but also would be informative to elucidate evolutionary processes that gave rise to these interspecific patterns. This also applies to metabolic rate scaling in general, where the functional basis for scaling theories appears to depend on the evolutionary time scale under investigation. These approaches would help to bridge the gap between micro and macrophysiology.
LITERATURE CITED


Weis-Fogh, T., (1964a) Diffusion in insect wing muscle, the most active tissue known. J. Exp. Biol. 41, 229-256.


STATEMENT OF CO-AUTHORSHIP

Chapter 4 of this thesis includes text modified from the following previously published articles:


This signature of my supervisor confirms my assertion that the allometric cascade proposed hypothesis, data analysis, and conclusions in the articles were primarily my own, worked in collaboration with my supervisors, the late Peter W. Hochachka and Raul K. Suarez, and co-author Russell D. Andrews.