



uOttawa

L'Université canadienne
Canada's university

**FACULTÉ DES ÉTUDES SUPÉRIEURES
ET POSTDOCTORALES**



**FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES**

Dimitri Ariel Skandalis

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Epidemiology)

GRADE / DEGREE

Department of Epidemiology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Contributions of Life History and Inter-Individual Variation to the Study of Energetic Supply and Demand in *Bombus Impatiens*

TITRE DE LA THÈSE / TITLE OF THESIS

Charles-Antoine Darveau

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

John Lewis

Jean-Michel Weber

Jeff Dawson

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

**THE CONTRIBUTIONS OF LIFE HISTORY AND INTER-INDIVIDUAL
VARIATION TO THE STUDY OF ENERGETIC SUPPLY AND DEMAND IN
*BOMBUS IMPATIENS***

Dimitri A. Skandalis

A Thesis submitted to the Department of Biology
in partial fulfillment of the requirements of M.Sc.

University of Ottawa

Ottawa, Ontario

© Dimitri Ariel Skandalis, Ottawa, Canada, 2011



Library and Archives
Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file *Votre référence*
ISBN: 978-0-494-79680-1
Our file *Notre référence*
ISBN: 978-0-494-79680-1

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.


Canada

ACKNOWLEDGEMENTS

I am immensely pleased to have too many people to thank. Foremost, I am very privileged to be able to thank my family -Paula, Mom, Dad- and scientific advisory committee in the same breath. I thank my supervisor and editor, Dr. Charles Darveau, for funding the research and for asking the questions that I was hoping to avoid having to ask. I doubt whether the project would have been possible at all without the material support, training, and the patience of Dr. Catherine Plowright and her students, Emily Sibbald and Levente Orbán. I will return your forceps soon. I have had so many important discussions with so many people, and learned so much, that the outcome of this could not have been predicted at my start. I am grateful for my experience in the laboratory and seminars of Dr. Jeff Dawson, which has greatly influenced my understanding of insect flight. Dr. Antoine Morin was a statistically significant factor in the analyses. Dr. Glenn Tattersall was a source of encouragement and support, and often-voluntary loans of equipment. Sometimes a whole research path can arise from a single lunch conversation, as happened with Dr. Jean Richardson. The same project in another environment might have had a very different outcome. I am grateful to all the members of the Comparative Physiology Group and Macroecology Group at uOttawa, for listening to me and fueling ideas and approaches that I would never have thought of on my own.

TABLE OF CONTENTS AND PAGE NUMBERS

Lists of Figures and Tables	5
Lists of Abbreviations	7
List of Abbreviations of Enzyme Names	8
Abstract / Résumé	9
Chapter 1: The study of intra-specific variation and phenotypic plasticity	10
Chapter 2: Behavioural, morphological, and metabolic maturation of newly emerged adult workers of the bumblebee, <i>Bombus impatiens</i>	
Abstract	17
Introduction	17
Methods	19
Results	24
Discussion	26
Chapter 3: Metabolic organisation in worker bumblebees (<i>Bombus impatiens</i>) I: covariation and plasticity of flight physiology	
Abstract	38
Introduction	39
Methods	42
Results	47
Discussion	53
Chapter 4: Metabolic organisation in worker bumblebees (<i>Bombus impatiens</i>) II: biochemical plasticity and metabolic covariation	
Abstract	78
Introduction	79
Methods	82
Results	84
Discussion	87
Chapter 5: Ecological and evolutionary consequences of plasticity and variation	99
Literature Cited	117

LIST OF FIGURES AND PAGE NUMBERS

Figure 2.1 Time courses of wing unfurling and development of struggling wing beat frequency.	33
Figure 2.2 Age at first observation of bees making guarding sorties out of the colony.	34
Figure 2.3 Mean time course of raw enzyme maturation data and fitted data obtained from the logistic regression.	35
Figure 2.4 Representative time courses of enzyme maturation after emergence in <i>Bombus impatiens</i> .	36
Figure 3.1 Correlation of total body mass to flight metabolic rate, water loss rate, wing beat frequency, and thorax temperature.	68
Figure 3.2 Influence of body size idiosyncrasies on wing beat frequency during flight.	69
Figure 3.3 Comparison of absolute and body mass-independent correlations of f_{flight} and metabolic rate.	70
Figure 3.4 Covariation of thorax temperature with metabolic rate and wing beat frequency.	71
Figure 3.5 Graphical summary of the direct and mass-independent correlations among measures of individual body size and physiology.	72
Figure 3.6 Undirected dependency graph obtained from the partial and mass-independent partial correlations among measures of individual body size and physiology.	73
Figure 3.7 Effect of flight experience on metabolic rate, wing beat frequency, and thorax temperature.	74
Figure 3.8 Distribution of lifetime flight experience and its impact on metabolic rate.	75
Figure 3.9 Effect of induced wing asymmetry on metabolic rate, wing beat frequency, and thorax temperature.	76

Figure 4.1 Direct and partial correlations between body size and metabolic enzyme activity.	93
Figure 4.2 Correlation of hexokinase activity to thorax mass.	94
Figure 4.3 Mass-independent correlations of hexokinase to other enzymes' activities.	95
Figure 4.4 Hexokinase activity does not change with flight history.	96
Figure 4.5 Flux velocity through hexokinase as a function of body mass and thorax temperature.	97
Figure 4.6 Correlations between body size, flight physiology, and enzyme activities.	98
Figure 5.1 Relationship of lifetime flights to age at first flight.	114
Figure 5.2 Hypothesised directional paths among flight variables in this study.	115
Figure 5.3 Variation in temperature-dependent hexokinase (HK) activity over the range of thorax temperatures found within the population.	116

LIST OF TABLES AND PAGE NUMBERS

Table 2.1 Parameters of post-emergence enzyme maturation in <i>Bombus impatiens</i> workers.	32
Table 3.1 Body mass and colony effects on parameters of body size parameters.	63
Table 3.2 Mean values and mass-scaling scaling exponents of for size and flight parameters.	64
Table 3.3 Main and interaction effects on flight parameters.	65
Table 3.4 Effects of flight restriction on flight phenotypes.	66
Table 3.5 Effects of wing cutting on flight phenotypes.	67

LIST OF ABBREVIATIONS

Abbreviation	Variable	Units
f_{flight}	Wing beat frequency in flight	Hz
f_{struggle}	Wing beat frequency during struggle response	Hz
M_{body}	Mass of whole body	mg
M_{thorax}	Mass of thorax	mg
M_{muscle}	Mass of flight muscle	mg
MR	Metabolic rate	mL O ₂ hr ⁻¹
SA_{planform}	Surface area of wing planform	mm ²
SA_{FW}	Surface area of forewings	mm ²
SA_{HW}	Surface area of hindwings	mm ²
T_{thorax}	Temperature of thorax	°C
T_{head}	Temperature of head	°C
T_{abdomen}	Temperature of abdomen	°C
T_{50}	Time to 50% enzyme maturation	hrs
t_e	Time of emergence	0 hrs
V_{max}	Enzyme activity	U g thorax ⁻¹
V_{mature}	Enzyme activity of mature adults	U g thorax ⁻¹
V_1	Relative V_{max} at emergence	%
$V_{48\text{h}}$	Enzyme activity at 48 hrs	U g thorax ⁻¹
V_{CO_2}	Metabolic rate (carbon dioxide release)	mL CO ₂ hr ⁻¹
$V_{\text{H}_2\text{O}}$	Water loss rate	Pa H ₂ O hr ⁻¹

LIST OF ABBREVIATIONS OF ENZYME NAMES

Abbreviation	Enzyme
ALD	Aldolase
CS	Citrate synthase
ENOL	Enolase
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
α GPDH	α -Glycerol-3-phosphate dehydrogenase
GP	Glycogen phosphorylase
HK	Hexokinase
PFK	Phosphofructokinase
PGI	Phosphoglucose isomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PK	Pyruvate kinase
TPI	Triosephosphate isomerase
TRE	Trehalase

ABSTRACT

In insects, flight energetics varies greatly among species but the source of this variation remains unknown. This thesis addresses phenotypic plasticity and matching of cellular and whole-animal metabolism in a bumblebee (*Bombus impatiens*). Bumblebees begin flying prior to full metabolic maturation, suggesting a window of greater metabolic plasticity. Bees were induced to fly before fully maturing, but although flight training resulted in elevated metabolic rates compared to controls, there was no change in biochemical activities of any tested enzymes. Similarly, while statistical models of flight parameters explained the majority of inter-individual variation in measures such as metabolic rate and wing beat frequency, there was no direct link between whole-animal metabolism and cellular energy production pathways. This suggests that muscle energy production and whole-animal metabolic demands are not plastically synchronised, and that inter-specific variation is likely not solely the result of phenotypic plasticity.

RÉSUMÉ

Chez les insectes, l'énergétique du vol varie de façon importante entre les espèces, mais les sources de cette variation demeurent inconnues. Cette thèse porte sur la plasticité phénotypique et le lien entre le métabolisme cellulaire et l'animal entier chez le bourdon (*Bombus impatiens*). Les bourdons commencent leur vol avant que la maturation métabolique soit terminée, suggérant une période de plasticité métabolique. La manipulation de l'effort du vol a augmenté le taux métabolique, mais aucun changement de l'activité des enzymes étudiées n'a été observé. Également, des modèles statistiques expliquent la majeure partie de la variation interindividuelle de mesures telles que le taux métabolique et la fréquence de battement d'ailes lors du vol. Cependant, aucun lien direct entre le métabolisme de l'animal et les voies de production énergétique n'a été établi. Ces résultats suggèrent l'absence de synchronisme des propriétés musculaires et du métabolisme de l'animal, limitant le rôle de la plasticité phénotypique.

CHAPTER 1:
THE STUDY OF INTRA-SPECIFIC VARIATION AND PHENOTYPIC
PLASTICITY

Over the years it has become clear that adjustments to the physical environment are behavioral as well as physiological and are inextricably intertwined with ecology and evolution. Consequently, a student of the physiology of adaptation should not only be a technically competent physiologist, but also be familiar with the evolutionary and ecological setting of the phenomenon that he or she is studying.

George A Bartholomew (1987)

Before there was integrative biology, there was organismal physiology. Uniquely among the fields of biology, physiology seeks to integrate organismal functioning with stressors, environments, and behaviours. Organismal adaptation and acclimation are not limited to any of the defined levels of (animal) biology, so physiologists study all levels, and borrow all techniques: genetics, biochemistry, behaviour, and physics, to name a few. Mangum and Hochachka (1998) reviewed the three philosophical approaches that are used by physiologists to gain insight. One approach, the comparison of genetic variation to phenotypic variation, has been highly successful. In insects, this led to the identification of the malate dehydrogenase and phosphoglucose isomerase genotypes as important determinants of metabolism and performance, in bees and butterflies respectively (Harrison *et al.* 1996a; Niitepõld 2010). Genetic variation has also been mapped onto metabolic variation using quantitative trait loci (Montooth *et al.* 2003). Perhaps most common is the comparative method, which has led to important insights into correlated evolution of biochemical supply and whole-animal metabolism, and evolutionary limits to design and performance (Clark & Wang 1994; Darveau *et al.* 2005b; Iwamoto *et al.* 2006; Pierce & Crawford 1997; Suarez 1996).

The third, and ‘untapped’ resource is the use of variation between individuals to discover the components that confer greater ability. For instance, Garland (1984) found that in the lizard *Ctenosaura similis*, individuals’ performances during endurance exercise (e.g., duration and peak metabolic rate), were strongly predictable by variation in enzyme activities and organ masses. Intra-specific variation can shed light on evolutionary patterns. Darveau *et al.* (2005b) examined interspecific variation between orchid bee species (Apidae: Euglossini) and found that mass-specific metabolic rate, wing beat frequency, and one enzyme among those studied, hexokinase, all scaled with body mass (M_{body}) with strikingly similar exponents ($\sim M_{\text{body}}^{-0.30}$). How much of this pattern was the result of correlated evolution of metabolic supply and demand, and how much was intra-specific plasticity? Lower wing beat frequencies at larger body sizes is a common intra-specific pattern in bees (e.g., Joos *et al.* 1991; Roberts *et al.* 2004). Intra-specific biochemical variation can also predict interspecific differences. Clark and Wang (1994) examined both inter-specific variation in enzyme activities and energy stores in the genus *Drosophila*, and compared this to intra-specific variation in *D. melanogaster*. They found that interspecific patterns were frequently predictable from the underlying intra-specific variation. For instance, species that stored large amounts of triacylglycerol also stored large amounts of glycogen, as predicted intra-specifically. Therefore, some inter-specific patterns in correlated variation could directly result of intra-specific plasticity. Tests of this hypothesis are few, but have profound implications for the relative contributions of plasticity and genetics to interspecific variation. Returning to the orchid bee study (Darveau *et al.* 2005b), the startling inter-specific coordination between physiological components could be the result of an underlying ‘rule’ specifying the integration of morphology and physiology, common among all bees. One such rule could be genetic specification of the slope of, e.g., enzyme capacity on body size.

Alternatively, muscle could be purely plastic, responding to any change in its local environment via feedback from metabolic requirements. By analogy, if muscle were transplanted from one bee to another, the muscle would morph to match the metabolic requirements of the new body. These two cases can be distinguished by testing for intra-specific phenotypic plasticity in response to demand. If changes in metabolic requirements do not plastically alter phenotypes, then it is likely that most specification occurs genetically.

Therefore, the aims of this study are three-fold. First, I examine the maturation of muscle, and particularly its time course with respect to the age at which flight is first initiated (Chapter 2). In many species, there is a delay between first flights and full maturation, which suggests a window during which muscle may plastically respond to demand. Second, I examine to what degree the patterns observed within a species resemble those found between species (Chapters 3 and 4). Intra-specific studies with large sample sizes and low genetic variation allow one to examine the sources of inter-individual variation. After accounting for the majority of variation in physiological parameters caused by idiosyncrasies, such as individuals with relatively larger or smaller wings, what remains is the phenotypic plasticity caused by a treatment or environmental condition.

There are, I think, two issues with intra-specific studies that have prevented their general adoption. Inter-individual comparisons are probably most useful when the mechanistic and adaptational underpinnings of the question have been broadly studied, like evolutionary physiology in general (Mangum & Hochachka 1998). Without this background, it can be difficult to understand the meaning of the results. To gather meaningful data, intra-specific studies demand a large volume of data, which has been a barrier. For studies that attempt to bridge and integrate multiple biological levels, this compounds the possible sources of error, from equipment to uncooperative subjects. This

latter problem has been greatly assuaged in the last decade by the development of high-throughput and high-precision research equipment, including flow-through respirometry, plate spectrophotometers, and microarrays. For the first time, it is possible to collect large sample sizes with large numbers of variables: physiology can become -omic. A grand challenge for physiologists (Mykles *et al.* 2010) will be to harness this capability in order to research variation between individuals, and vertical integration to link different levels of biology together.

Having collected the data, there is another, perhaps more daunting problem. All -omes are stymied by our ability to analyse the data. Between individuals, physiological parameters are highly correlated both with body size and with each other (Garland 1984; Clark & Keith 1988). This makes it difficult to ascertain if two variables are correlated with each other directly, or because of covariation to another parameter. This is why, generally, correlation does not imply causation. My approach in this thesis has been to collect a large amount of data, to ease comparisons of sources of variation, and then to visualise the data as the components that can be attributed to different causes. 'Cause' may incite opposition, and I will attempt to minimise its use until Chapter 5, the Discussion. However, Shipley (2002) points out that it is a fundamental tenet of research that causation does imply correlation. Where a causal link exists between two variables, we assume we can find it if we want to. Since the complication of finding this link is the possibility of covariation, I have attempted to collect data from a wide variety of sources. This includes multiple measurements of functionally similar parameters, such as morphology or temperatures of different body parts. By including these, I attempted to increase the resolution and find biologically meaningful correlations that imply causation, or can be tested further.

In the three data chapters of this thesis, I explore the integration of morphology, cellular biochemistry, and whole-animal flight performance, in sequence from new, maturing workers to flying adults. In Chapter 2, I ask how the initiation of flight in holometabolous insects, those possessing complete metamorphosis, is timed with respect to morphological and biochemical maturation. This helps to identify which parts of physiology may be 'rate-limiting' to flight performance, and how closely the bees may be operating with respect to biochemical matching of energy supply to demand (e.g., Staples & Suarez 1997). Chapters 3 and 4 attempt to dissect patterns in the physiology of mature adults. Building on the matching of energetic supply and demand, I look for morphological and physiological differences between individuals that help explain why they perform differently in similar situations. The result is a set of patterns that identify how a core set of flight variables interact in relatively constant conditions. Finally, I discuss these results in an ecological context, and speculate on how inter-individual variability may have further consequences that I have not studied. I hope these results serve as a basis for identifying how integration in biology develops and is maintained, and the environmental or behavioural conditions under which new patterns develop.

CHAPTER 2:
BEHAVIOURAL, MORPHOLOGICAL, AND METABOLIC MATURATION OF
NEWLY EMERGED ADULT WORKERS OF THE BUMBLEBEE, *BOMBUS*
IMPATIENS

ABSTRACT

Newly emerged adult holometabolous insects must still complete considerable morphological, metabolic, and neural maturation. Despite this, adults have frequently been documented to fly prior to attaining maximum capacity. In some species, flight is limited by the unfurling of the wing, while in other species it may be limited by biochemical capacity. I charted maturation trajectories of adult bumblebee workers (*Bombus impatiens*) for both morphological and enzymatic parameters, and compared these to the first age at flight. Workers begin regular flights two days after emergence. The unfurling and hardening of the wings is completed before any other studied component, and before flight, suggesting this does not initially limit flight. Wing beat frequencies, measured as a struggling response to grasping the hindlegs, matured on a time course more similar to flight. By the initiation of flight, the mean enzyme maturation was only 50% completed relative to adult enzyme capacity, though specific enzyme profiles ranged from 30% to 75%. Bumblebees, as other adult insects, thus begin flights prior to fully maturing. On this basis, I suggest that maturing insects may be a useful future tool for examining the consequence of reduced biochemical capacity on safety margins and regulation of whole-animal flight physiology.

INTRODUCTION

In order to prepare for energetically intensive flight, metamorphosing insects histolyse the weak larval muscles and replace them with new and more powerful ones (Fernandes *et al.* 1991). Maturation of the new flight muscles usually continues even after emergence of the new adults, resulting in an overlap between physiological maturation and the onset of flight behaviour. Due to this overlap, early flight competency may be reliant on

ongoing changes in muscle properties such as enzyme content (Beenakkers *et al.* 1975), mitochondrial volume (Herold 1965), and muscle elasticity (Fielding *et al.* 1980).

In some cases, the flight of newly emerged adults is dependent only on the unfurling and hardening of the wings, such as in the butterfly *Pieris* (Petersen *et al.*, 1957). In other species, the initiation of flight may be delayed by the maturation of enzyme capacities. Among six insect species, initial α -glycerol-3-phosphate dehydrogenase (α GPDH) activities in new adults ranged from 2.5-68% of the eventual adult maximum, and higher initial activities predicted earlier ages of first flights (Campbell & Birt 1972). Though Campbell & Birt (1972) suggested that α GPDH alone predicted the start of flight, which enzymes mature, and so which may be initially limiting, is dependent on the flight fuel source of the adults (Beenakkers *et al.* 1975).

Intriguingly, many insects are capable of flight prior to achieving full biochemical or mechanical competence. Honeybee workers (*Apis mellifera*) begin their first orientation flights well before they begin foraging (Herold & Borei 1963), and prior to attaining maximum enzyme capacity (Herold & Borei 1963; Harrison and Fewell 2002). Biochemically immature blowflies (*Phormia terraenovae*) males start flying even though some enzymes only have 40% capacity (Collatz *et al.* 1981). After emergence, enzyme trajectories frequently mirror life-history transitions. The loss of glycolytic enzyme capacity corresponds to the loss of flight in *P. terraenovae* males (Collatz *et al.* 1981). The conversion from nursing to foraging behaviour in honeybee workers coincides with upregulation of enzymes involved in heavy work and high metabolism (Wolschin & Amdam 2007; Schippers *et al.* 2006). The regulation of metabolism is therefore a dynamic activity in the lifetime of the adult insect, and not necessarily permanently established at emergence. Dynamic regulation of metabolism has important ecological and evolutionary

consequences, since organisms which have greater ability to acclimatise to local environmental conditions may have less drastic selection pressures for adaptation to the same conditions. In particular, the ability to fly prior to full maturation suggests a period in which flight experience might induce plastic changes in energetic supply in response to demand. In order to further study whether such a window might affect the phenotypic plasticity of the flight muscles (Chapters 3 and 4), I examined how morphological and biochemical maturation profiles compare to the initiation of flight in bumblebee workers (*Bombus impatiens*). *Bombus impatiens* is endemic to North America and raised as a pollinator for greenhouses. New workers are distinguished by their high degree of wing curvature and their silvery grey pile which yellows in about 24 hrs. Heinrich (1979) estimated that new workers begin flying around 48 h after emergence, and I therefore focused on characterising the maturation of glycolytic enzymes from 0-48 h intensively, and daily thereafter. I examined the complete suite of glycolytic enzyme activities to examine their coordination during maturation. I also examined changes in activities of enzymes involved in substrate recruitment (glycogen phosphorylase and trehalase), and those which shunt NAD-cofactors to other biochemical paths (glucose-6-phosphate dehydrogenase and α GPDH). Finally, citrate synthase was studied as a representative of the tricarboxylic acid cycle and mitochondrial maturation.

METHODS

Housing colonies

Colonies of *B. impatiens* were obtained from a local greenhouse supplier (BioBest Canada, Ltd) and housed in their shipping box. I maintained ambient temperatures at 25-26°C with a 16:8 light-dark cycle, though the colony is almost completely dark when the box

is closed. Bees were fed *ad libitum* sugar water and pollen balls (unlyophilised), presented directly to the colony.

New workers were collected daily while their silver pelosity distinguished them from mature adults. Each individual was marked with a coloured and numbered card disc, glued between the tegulae with resin glue, and then replaced in the colony. Individuals' ages were recorded as the time in hours post-emergence from their cocoons. Data for the age at first flight was obtained from colonies held in free-flight enclosures. Colonies were stimulated daily by shaking, which resulted in workers performing guarding sorties (experiment described in Chapter 3). Flying guards were captured, and their identities recorded. The age at first flight was the first day an individual was observed. I observed similar results if the stimulus to leave the colony was foraging at a nectar source instead.

Collection of individuals

The physiological maturation of workers was studied in two phases. A fine time scale of measurements was made with individuals aged 0, 3, 6, 12, 18, 24, 48 and >180 hrs post-emergence. The age at marking of these bees was controlled to ensure representativeness of the data. The ages of bees collected for time intervals between 0-6 hrs old were known exactly, as they were taken while emerging from their cocoon. Otherwise, ages of individuals collected between 12-24 hr old were known to within 1 hr; ages of individuals collected at 48 hr were known to within 3h; ages of individuals collected after 180 hr or older were known to within 24 hrs. A second set of measurements was made on a coarser time scale, using individuals collected at ages from 0-6d old. 0 d old individuals were collected as they emerged from their cocoons, and otherwise ages were known to

within 12hrs. For data on the age at first flight, individuals' ages were only known to within 24 hr.

Workers' wings were photographed in profile, and analysed in ImageJ software (Rasband, 1997-2009) to find the time course of wing unfurling. A dimensionless wing curvature coefficient was found by dividing the area under the wing, from tegula to tip, by the square of the wing length. Maturation of wing beat frequency (f_{struggle}) was tested in a group of males tested repeatedly, though haphazardly, over the first five days after emergence. Immature adults cannot fly, so f_{struggle} was acquired by grasping a hind limb with a pair of forceps, inducing a struggling wing beat response (which is correlated to an individual's wing beat frequency in flight, Chapter 4). Beat frequency was measured by an optical tachometer (Moore Scientific) and Trex 2.0 Transient Waveform Recorder.

Enzyme assays

Enzymes are expressed as their maximum activity *in vitro* (V_{max} ; U g thorax⁻¹). Six or 24 bees were analysed for enzyme activity daily. Frozen thoraces were weighed and then homogenised in 19 volumes of ice-cold homogenisation buffer (25mM Tris-KPO₄, pH 7.8 at 4°C, 2mM EDTA, 5mM DTT and 0.5% Triton X-100), or 1000µL, whichever was less. This prevented overflow during the automated homogenisation procedure. Homogenisation was performed in three 10s bursts with 30s intervals (six 5mm heads, 10,000 RPM, OMNI-Prep Homogenizer), and the homogenate was then sonicated in two 5s bursts with 20s intervals, at 20% amplitude (Sonics Vibra-cell, six heads). Homogenates under 1000µL were then brought to volume, vortexed, and spun down at 500g for 10 mins. The supernatant was tested for activities.

Enzyme assays were optimised with respect to reagent concentration and performed at the *in vitro* pH optimum, and at 37°C. Each homogenate was assayed in triplicates of 10µL diluted homogenate in 240µL of reaction buffer, on a BioTek Synergy2 plate spectrophotometer (Winooski, VT). We report the best duplicate enzyme activities in U g thorax⁻¹, where 1U=1 µmol substrate min⁻¹ (substrate is NAD-cofactor or DTNB). All reagents were obtained from Sigma except Acetyl-CoA (BioShop Canada Inc) and ATP (Calbiochem). Protocols for TRE, HK, PGI, CS, and αGPDH were modified from Darveau *et al.* (2005b); ENOL and PK from Pierce and Crawford (1994); G6PDH from Joannis and Storey (1994). Coupling enzyme was added at 1.25U/250µL, except where noted. Assay conditions, with pH given at 37°C, are as follows: Glycogen phosphorylase (GP): 100mM phosphate (pH 7.1), 10mM MgCl₂, 4µM G1,6P₂, 0.75mM NADP, 2mM AMP, 1mg/250µL glycogen, phosphoglucose mutase and G6PDH. Trehalase (TRE): 100mM phosphate (pH 6.6), 1.1mM MgCl₂, 0.75mM NADP, 1.1mM ATP, 10mM trehalose, HK and G6PDH. Glucose-6-phosphate dehydrogenase (G6PDH): 50mM Tris-Imidazole (pH 7.5), 10mM MgCl₂, 0.1mM NADP, 1mM G6P. Hexokinase (HK): 100mM Tris-Imidazole (pH 8.1), 100mM KCl, 10mM MgCl₂, 1mM NADP, 5mM ATP, 5mM D-glucose, G6PDH. Phosphoglucose isomerase (PGI): 50mM Tris-Imidazole (pH 7.8), 5mM KCl, 10mM MgCl₂, 0.75mM NADP, 16mM F6P, G6PDH. Phosphofructokinase (PFK): 50mM Tris-Imidazole (pH 8.0), 100mM KCl, 10mM MgCl₂, 0.2mM NADH, 2mM ATP, 10 µM F2,6P₂, 5mM F6P, Aldolase, TPI, αGPDH. Aldolase (ALD): 100mM Tris-Imidazole (pH 7.0), 10mM KCl, 0.15mM NADH, 1.5mM F1,6P₂, TPI and αGPDH. α-Glycerol-3-phosphate dehydrogenase (αGPDH): 50mM Tris-Imidazole (pH 6.1), 0.15mM NADH, 1mM DHAP. Triosephosphate isomerase (TPI): 100mM Tris-Imidazole (pH 6.1), 10mM KCl, 0.15mM NADH, 4.8mM G3P, αGPDH. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH): 100mM Tris-

Imidazole (pH 6.7), 10mM KCl, 2mM MgCl₂, 0.15mM NADH, 3.1mM ATP, 10mM 3PG, PGK. Phosphoglycerate phosphokinase (PGK): 100mM Tris-Imidazole (pH 6.4), 10mM KCl, 5mM MgCl₂, 0.15mM NADH, 3.1mM ATP, 28mM 3PG, G3PDH. Phosphoglycerate mutase (PGM): 100mM Tris-Imidazole (pH 6.8), 10mM KCl, 5mM MgCl₂, 0.15mM NADH, 1.25mM ADP, 2,3-BPG, 9mM D-glucose, 2.8mM 3PG, ENOL (0.025U/250μL), PK, lactate dehydrogenase (LDH), and HK. Enolase (ENOL): 100mM Tris-Imidazole (pH 6.5), 10mM KCl, 5mM MgCl₂, 0.15mM NADH, 1.3mM ADP, 9mM D-glucose, 2mM 2GP, PK, LDH, and HK. Pyruvate kinase (PK): 100mM Tris-Imidazole (pH 8.1), 10mM KCl, 5mM MgCl₂, 0.15mM NADH, 0.4mM ADP, 0.75mM F1,6P₂, 5mM PEP, LDH. Citrate synthase (CS): 50mM Tris-HCl (pH 7.4), 0.3mM Acetyl-CoA, 0.1mM DTNB, 0.5mM OA. Citrate synthase activity was followed at a wavelength of 412nm and extinction coefficient of 13.6. All other enzymes were followed at 340nm and 6.22, respectively. I corrected for a path length of 0.705 cm.

Data analysis

All analyses were performed in the R language environment (v 2.10, R Development Core Team 2009). Partial F- or *t*-statistics are reported with degrees of freedom in subscript. For comparisons of linearly related variables, sequential anova (type I sums of squares) was used ($\alpha=0.05$).

I examined the trajectory of enzyme maturation with age, first as a function of individuals' mean enzyme content, and then I examined each enzyme separately. In order to obtain a mean profile of enzyme maturation, age-specific enzyme activities were normalised to the mean of the mature adult V_{\max} (V_{mature}) of that enzyme. The mean profile is then calculated from the mean of each individual's enzymes, excluding G6PDH. Maturation of

most components in this study was nonlinear with age, so nonlinear least-squares regression was performed with a self-start fitting process and the function `nls()`. This process guesses initial parameters for the regression model, and then converges on the best parameters based on minimising the Akaike Information Criterion. The fitting process, and thus the derived parameters, may be prone to the distribution of the data. To compensate, parameter errors for each enzyme were obtained by resampling 4/5 of the data points and recomputing parameters in 1000 iterations. The data are then provided as means and standard deviations of the 1000 resamplings.

For each enzyme, I calculated its initial activity at emergence (V_i), its time to 50% completed maturation (T_{50}), its activity at 48 hrs (V_{48h}), and its activity in mature adults (V_{mature}). V_i is reported as the V_{max} of individuals between 0-6 hr ($n=12$) except for G6PDH where only 0 hr individuals were available ($n=4$). V_i is expressed as percentage of V_{mature} . T_{50} is obtained from the inflection point of the modeled logistic regression, and the enzyme activity at 48h (V_{48h}) was calculated from the model fitting parameters.

RESULTS

Behavioural and morphological maturation

The immature adult's wings were initially highly curved, primarily in the distal portion. The wings completely unfurled between the first and second day post-emergence (t_e) from the cocoon, though some curvature was always present (Fig. 2.1a). The gain of wing beat frequency in males ($f_{struggle}$) followed a slower time course than the unfurling of the wings (Fig. 2.1b). At t_e , male $f_{struggle}$ was ~ 80 Hz, and increased logarithmically with age ($r=0.989$) until reaching a maximum of ~ 160 Hz at 3-4 d post- t_e .

Workers began guarding sorties 2 d after emergence (Fig. 2.2). Although a few individuals were recorded performing guarding sorties at 1 d old, this was probably because ages were only known to within 1 d in that experiment. I examine the development of flight capability more closely by provoking involuntary flights in workers. Workers were dropped from ~1 m. At 0 d post- t_e , the bee could not control her descent, but by 1 d post- t_e she was capable of slowing and directing her fall by beating her wings. At 2d post- t_e , individuals were fully capable of flight. In a separate experiment I saw that workers began voluntary foraging flights to a nectar feeder at 2 d old (data not shown).

It is possible that the flight muscle mass increases with age, reflecting protein synthesis and increases in the mass of the myofibrils. I did not measure flight muscle mass directly, and so compared thorax mass (M_{thorax}) across individuals (muscle mass is ~89% of M_{thorax} , Buchwald & Dudley 2010). Morphology does not change with age in holometabolous insects, and in the mature adults of this study, the wing planform area was highly correlated with M_{thorax} ($F_{1,26}=786.1$, $p<0.001$). I therefore used wing area to control for differences in body ‘size’ while comparing M_{thorax} to age; there was no change in M_{thorax} with age ($F_{1,36}=0.39$, $p=0.53$).

Biochemistry

I first found the average activity of each enzyme in mature adults (Table 2.1), in order to compare relative content at different ages. I then compared how enzyme activity changes, on average, with age. Both enzyme activity and age were log transformed to better differentiate changes at younger ages. The mean profile increased logistically with age (Fig. 2.3), but initial logistic fits poorly characterised the data. I therefore added covariates to allow for variation in M_{thorax} and the day of biochemical assay. The resulting fit matched the

data with $R^2=0.98$ (compare fitted and raw data in Fig. 2.3), and so this process was retained when examining each enzyme separately. The lifetime variability in enzyme content, estimated as the model residuals divided by the fitted values, did not change with age ($F_{1,70}=0.03$, $p=0.87$).

Overall, enzyme content did not change over the first 6 h post-emergence, and was initially ~24% of its eventual maximum (considering ages ≤ 6 h together, $n=15$). At 12 h, activity began to increase, and at ~48h post-emergence individuals achieved the midpoint of maturation, with 63% of eventual enzyme capacity (significance of midpoint according to t -test: $t_{62}=63.62$, $p<0.001$).

Specific enzymes' profiles differed from the mean time course, indicating enzymes mature at different rates (HK, PGI, and TPI are shown in Fig. 2.4). The logistic fitting process was obtained for each enzyme except G6PDH and α GPDH, for which there was insufficient data. Initial activity at emergence, T_{50} and V_{48h} for each enzyme are collected in Table 2.1. Some enzymes reached their T_{50} sooner than did the mean profile, such as HK (Fig. 2.4a). Other enzymes reached their midpoint later, such as TRE and TPI (Figs. 2.4b and c). By 48 hrs after emergence, and the start of flight, three enzymes, PGI, PFK, and TPI, had not achieved at least 50% activity. Otherwise, activities at 48 h ranged to ~75% of V_{mature} (Table 2.1).

DISCUSSION

Bombus impatiens workers matured quickly after emergence, and were able to sustain flight by two days of age. This is seen in holometabolous insects such as Lepidoptera. Petersen et al. (1957) found that individuals of *Pieris napi* and *P. bryoniae* can fly within hours of emerging, after the wings have hardened, but noted that full behavioural

competency was attained some days later. In contrast, Gunn and Gatehouse (1988) found that enzyme capacity finishes maturing at least 24 hrs later (in *Spodoptera exempta* and *Mythimna separata*). In *B. impatiens*, workers between one and two days old could not sustain flight, even though their wings were mostly, or fully, unfurled (Fig. 1a). However, they were capable of slowing and directing their descent from elevation (Fig. 1b). Elevated wing beat frequencies (indicated by f_{struggle}) in males developed more slowly, to a maximum about three days post emergence (in males; Fig. 1b). By that age, workers can control their flight, and begin to fly and forage for nectar (Fig. 2; Heinrich, 1979; DAS, pers. obs.). A similar logarithmic increase in tethered flight wingbeat frequency occurs in maturing locusts (*Locusta migratoria*) and is related to neurodevelopment (Gray and Robertson, 1994). For the purposes of determining a rate-limiting step of maturation, it is impossible to distinguish whether wing beat frequency or biochemical capacity must mature first. On the other hand, it is likely incorrect to argue for a rate-limiting step at all, since the earliest ability to fly is probably determined by the state of the bee, i.e., taking into accounting wing beat frequency, biochemical capacity, etc., simultaneously.

The biochemical maturation of bumblebee workers was quick, similarly to honeybees (*Apis mellifera*). In both cases, completely mature glycolytic enzyme profiles appear three to five days after emergence (Fig. 2; Harrison, 1986; Schippers et al., 2010). Bumblebees and honeybees can begin voluntary flights around 3 d, but most honeybees begin foraging much later, around 10-20 days after emergence (Harrison, 1986; Harrison and Fewell, 2002; Herold and Borei, 1963). The difference in the age at first voluntary flights compared to foraging flights in honeybees cannot be due to glycolytic enzyme maturation, but it may be due to a delay in mitochondrial maturation. In bumblebees, maximum respiration on a combination of pyruvate, malate, and proline was reached three days after emergence (Roy,

2010), while in honeybees, maximal respiration on pyruvate and malate was reached at twenty days old (Balboni, 1967). This suggests that the start of regular foraging trips in older honeybees (Harrison, 1986) may be influenced by the gain of mitochondrial respiratory activity (Balboni, 1967), even while maturation of the glycolytic chain has been completed.

In general, enzyme activity appears to have coevolved with adult life history requirements (Campbell & Birt 1972; Darveau *et al.* 2005b). In most cases, age-specific matching of flight behaviours and biochemical activities is probably due to genetic coregulation. In some insects, experimentally manipulating behaviour does not result in altered enzyme activity, such as in the *P. terraenovae* life cycle (Collatz *et al.* 1981) and in *Musca domestica* (Chesky 1974). In a few cases, performance, or anticipation, of behaviour may impact muscle biochemical development. In honeybees, workers performing nursing or foraging behaviours have distinct muscle biochemical profiles, but forcing them to switch behaviours results in plastic switching muscle phenotype (Wolschin and Amdam 2007). However, this does not seem to be otherwise related to flight experience (Schippers *et al.* 2010), and so the change is anticipatory of flight performance, rather than dependent on it.

In comparison to programmed changes, examples in which muscle phenotype results directly from flight performance are rare (and rarely studied), but they suggest that a common factor may specifically be exercise during the maturational period. Tsetse flies (*Glossina morsitans*) show very significant increases in mitochondrial volume if they are exercised between blood meals (Anderson and Finlayson, 1976). *Drosophila melanogaster* restricted from flight throughout their lifetimes show reduced CS activity and altered membrane composition, but switching older flies back to the unrestricted treatment does not reverse the initial muscle development (Magwere *et al.*, 2006). However, when adult *L. migratoria* are exercised for long duration to recruit lipid oxidation, the increased delivery of

fatty acids directly stimulates a ten-fold increase in fatty-acid-binding protein (FABP) mRNA transcription (Chen and Haunerland, 1993). This highlights that more examples are needed to clarify the extent to which adult insect muscle can respond to exercise. Nonetheless, the maturational period is a good candidate for greater muscle plasticity, because mRNA transcription and protein translation only occur shortly after the emergence of the adult. For example, in locusts, FABP mRNA reaches a peak about the same time the protein concentration reaches half maximum, and then the mRNA rapidly declines while protein levels remain constant (Haunerland, 1997; van Marrewijk et al., 1980). In blowflies (*Lucilia sericata*), the ratio of RNA/protein, and concentrations of ribosomal- and transfer-RNA, peak in new adults, then rapidly decline (Holmes and Birt, 1977; Ring, 1973); simultaneously, ^{14}C -leucine incorporation rates peak as protein content of flight muscle increases, and then they decrease while protein levels remain constant in the mature adult (Bartelink et al., 1975). Since transcription and protein synthesis is most active in the maturational period, we had expected that G6PDH, which shunts glycolytic intermediates to ribose synthesis, would show initially high activity and then decrease in mature adults. We were surprised that G6PDH showed little change in the maturing bee, and indeed, slightly increased. Geer et al. (1972) also did not find a change in G6PDH activity in maturing *D. hydei*, even in the testis. It may not be possible to infer changes in ribose synthesis, or the period during which mRNA transcription is active, from G6PDH activity alone.

It is intriguing that insects initiate flight prior to full maturation because high enzyme capacities are thought to have evolved in insects to fuel their high mass-specific metabolic rates (Suarez, 2000). In mature adult honeybees, enzymes near equilibrium, such as PGI, operate very close to maximum forward velocity (Staples and Suarez, 1997). However, we observed that the bumblebees in this study began flying while some equilibrium enzymes'

activities had only achieved 30-50% of their mature maximum (Table 1). This could suggest many of these enzymes operate with considerable safety margins. Eanes et al. (2006) found that the wing beat frequencies of *D. melanogaster* in brief flights were generally highly resistant to knockdown of glycolytic enzyme activity. A notable exception was hexokinase (HK), so we compared the activity of HK in bumblebees to the amount of activity reduction in fruit flies required to alter wing beat frequencies. At the start of flight, bumblebee HK had matured to ~75% of capacity, but reduction to 67% HK activity did not affect flight performance in the knock-down flies (Eanes et al., 2006). Ideally, flight metabolic rates in maturing workers should be examined simultaneously with enzyme capacity. Otherwise, it is not possible to conclude if the bees are supporting flight with the same flight metabolic rates but lower enzyme capacities, or have lower flight metabolic rates altogether. The latter is probably the situation, since the maturation of honeybee flight metabolic rates parallel that of pyruvate and malate respiration (Balboni, 1967; Harrison, 1986).

Bees may be able to fly prior to maximum enzyme complement because they are not foraging or lifting loads, which increase metabolic rate (Feuerbacher et al., 2003). Moreover, excited foraging bees elevate thoracic temperatures, which also requires a higher metabolic rate (Moffatt, 2001; Mapalad et al., 2008). If this is the case, many enzymes show considerable safety margins, as much as 50% of some enzymes' mature activities. Alternatively, it is possible that enzyme capacity did not only coevolve with high mass-specific metabolic rates. In locusts, most metabolic maturation is completed prior to the gain of full flight competence, with the exception of FABP; the maturation of long-duration flights paces FABP maturation (Hauerland, 1997). Similarly, young blowflies with just 50% enzyme concentration can fly at speeds comparable to fully mature individuals, although they do not fly for long duration (Collatz et al., 1981). Flight duration is also

important for insects, and so sustained, rather than just instantaneous, high intensity power output may have been a factor in the evolution of high enzyme capacities.

The continuing maturational period in young adults may also have ecological relevance. Completing maturation after attaining some flight and environmental experience could impart a performance advantage in fluctuating environments. One way could be to allow individuals to plastically alter cellular metabolism in response to new information from the environment, which may not have been available while muscle was actually developing. The different hypotheses for the maturational period highlight that it should serve as a model for both ecological and evolutionary questions in integration and coordination of metabolism.

Table 2.1 Parameters of post-emergence enzyme maturation in *Bombus impatiens* workers.

For each enzyme, I present its initial capacity at emergence (V_i) as a percentage of the mature adult mean activity (V_{mature} ; U g thorax⁻¹), the mature adult V_{mature} , the enzyme capacity 48 hrs after emergence ($V_{48\text{h}}$), and the time to 50% completed maturation (T_{50}). Standard deviations (sd) of V_i and V_{mature} were calculated directly from the data (V_{mature} samples sizes, n , are indicated). Mean and error (ϵ) of T_{50} and $V_{48\text{h}}$ were calculated by resampling the data, as indicated in the text. Parameter estimates for G6PDH and α GPDH were not performed (nd).

Enzyme	$V_i \pm \text{sd} (\%)$	$V_{\text{mature}} \pm \text{sd} [n]$	$V_{48\text{h}} \pm \epsilon (\%)$	$T_{50} \pm \epsilon (\text{hr})$
GP	18.3 \pm 4.9	18.1 \pm 3.0 [32]	76.3 \pm 2.0	36.0 \pm 1.0
TRE	41.3 \pm 4.6	31.4 \pm 4.9 [31]	70.4 \pm 3.1	58.0 \pm 1.1
G6PDH	58.7 \pm 6.4	1.61 \pm 0.33[20]	nd	nd
HK	23.3 \pm 2.9	77.1 \pm 6.8 [32]	73.6 \pm 1.2	39.2 \pm 1.0
PGI	38.3 \pm 4.3	417 \pm 26 [31]	30.2 \pm 3.3	55.3 \pm 1.0
PFK	14.4 \pm 3.6	100 \pm 20 [8]	43 \pm 5.7	55.5 \pm 1.0
ALD	15.3 \pm 3.2	104 \pm 9 [32]	64.2 \pm 1.6	45.0 \pm 1.0
αGPDH	32.3 \pm 7.0	706 \pm 137 [7]	nd	nd
TPI	17.1 \pm 2.5	4490 \pm 530 [30]	32.6 \pm 3.3	71.7 \pm 1.0
GAPDH	19.5 \pm 5.6	388 \pm 21 [32]	63.6 \pm 2.1	46.6 \pm 1.0
PGK	38.8 \pm 9.3	842 \pm 69 [32]	70 \pm 7.2	47.8 \pm 1.0
PGM	27.4 \pm 5.3	787 \pm 83 [31]	75.1 \pm 1.8	40.2 \pm 1.0
ENOL	16.9 \pm 1.2	301 \pm 39 [32]	55.4 \pm 1.8	58.6 \pm 1.0
PK	15.7 \pm 2.4	223 \pm 14 [32]	56.7 \pm 1.2	52.3 \pm 1.0
CS	12.2 \pm 1.2	317 \pm 43 [32]	58.4 \pm 1.5	46.0 \pm 1.0

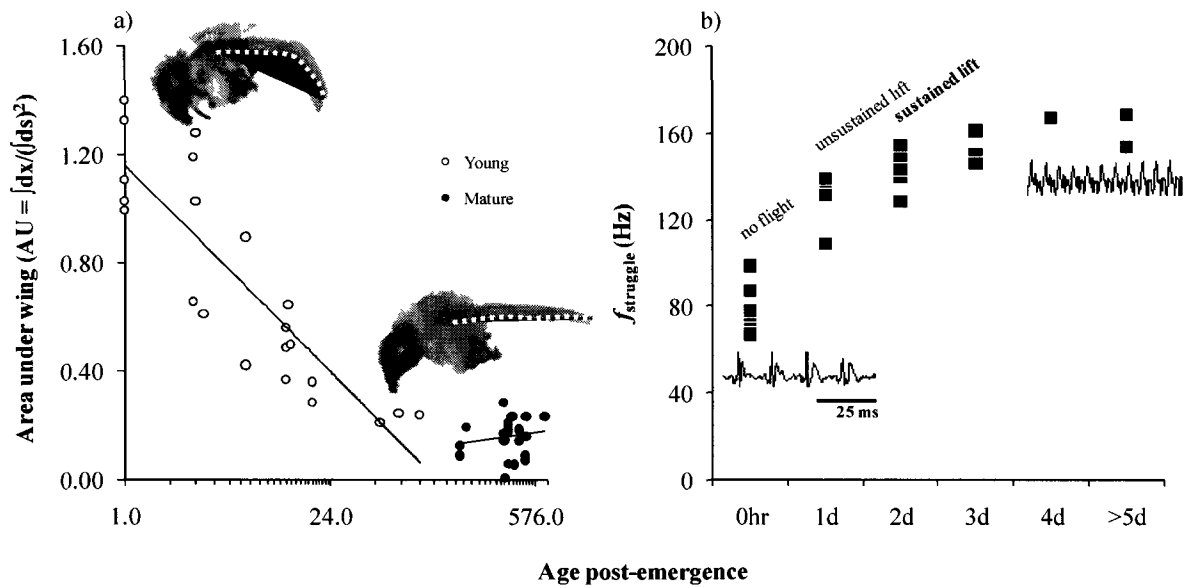


Figure 2.1 Time courses of wing unfurling (panel a) and development of struggling wing beat frequency ($f_{struggle}$, panel b). Ages in panel a are in hours. The curvature of workers' wings was assessed as the unitless ratio of the area under the wing (shaded black on inset) to the square of the length of the wing (stippled line on inset). The wing beat frequency ($f_{struggle}$) is measured as a male's struggling response to grasping his hindleg with forceps.

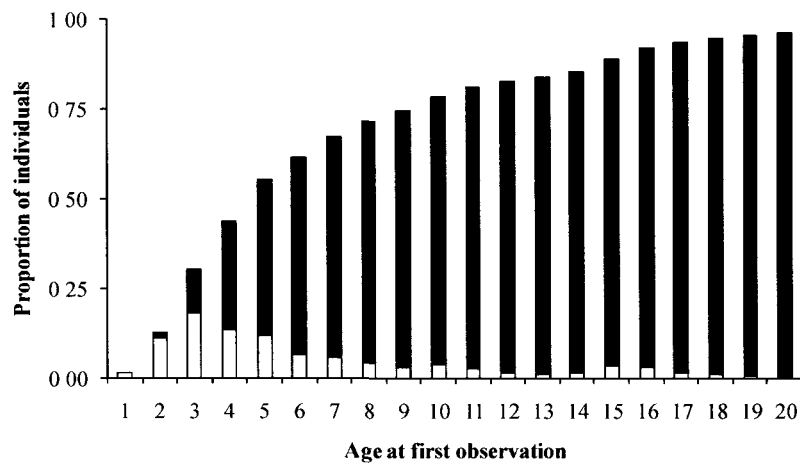


Figure 2.2 Age at first observation of bees making guarding sorties out of the colony. Information on individuals was collected once per day. White bars: unique observations; black bars: cumulative observations.

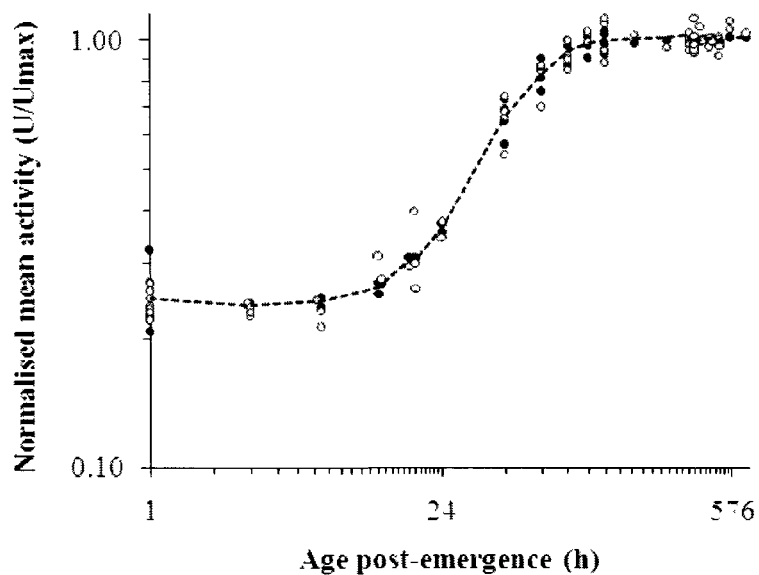


Figure 2.3 Mean time course of raw enzyme maturation data (white circles) and fitted data obtained from the logistic regression (black). The raw data points represent the mean maturation of all of an individual's enzyme activities, as a fraction of the activities of mature adults. Relative variation in enzyme content is constant over all ages.

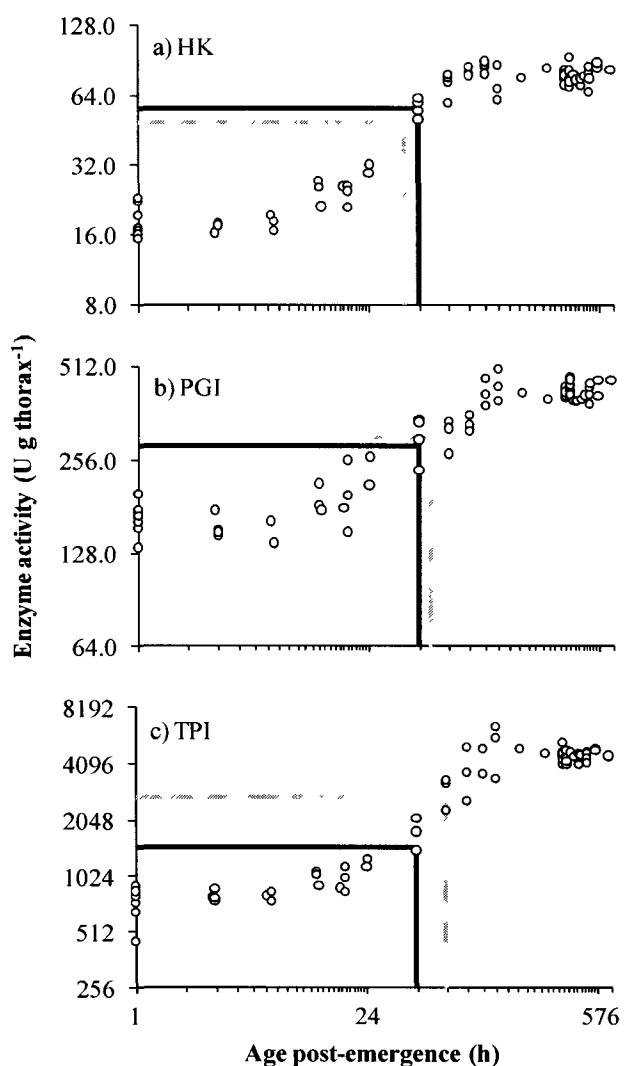


Figure 2.4 Representative time courses of enzyme maturation after emergence in *Bombus impatiens*. Panel a: Hexokinase (HK), panel b: phosphoglucose isomerase (PGI), panel c: triosephosphate isomerase (TPI). Data were fitted to a logistic function and then parameters extracted from the model. Black lines represent the enzyme activity at 48 hrs after emergence (V_{48h}), around the start of flight. Grey lines indicate the enzyme activity at the midpoint of maturation (T_{50}).

CHAPTER 3:
**METABOLIC ORGANISATION IN WORKER BUMBLEBEES (*BOMBUS*
IMPATIENS) I: COVARIATION AND PLASTICITY OF FLIGHT PHYSIOLOGY**

ABSTRACT

Within a species, individuals of similar mass show considerable variation in physiological parameters. One important cause of differences may be individual idiosyncrasies in development, such as larger or smaller wings than expected for a certain size, which lead to subtle downstream changes. Not all physiological parameters are necessarily directly related to body size, so it is a major challenge to understand how variation in one parameter impacts others. I explore this problem in the near-steady hovering flight of bumblebees, to understand how similar individuals differ in a similar environmental regime. By discriminating between direct and indirect correlations (given the parameters in the study), I develop a graphical interpretation of the correlations amongst flight variables, which I suggest models how different parameters contribute to inter-individual diversity. Total body mass best predicted metabolic rate (VCO_2), while wing surface area and thorax mass predicted wing beat frequency (f_{flight}). After accounting for their covariation with mass, VCO_2 and f_{flight} also explain significant variation in each other, but this is substantially weakened by covariation with the temperature of the thorax (T_{thorax}). The latter, measured at the surface in infrared, was only indirectly related to body size through f_{flight} and VCO_2 . Water loss rate was uniquely related to VCO_2 , and head and abdominal temperatures were separately and uniquely predicted by T_{thorax} . These results suggest paths in which flight phenotype is organised as a physiological cascade. I subsequently examined how manipulating flight might lead to correlated physiological changes, and whether these changes are supported by the inter-individual correlations. I first compared how flight experience alters performance, by restricting bees from flying throughout their lifetimes. This experiment corroborated the inter-individual patterns, and greater VCO_2 and f_{flight} predicted higher T_{thorax} in the experienced fliers. I then manipulated a parameter of body size

directly, by inducing wing asymmetry, to examine how this alters physiology. VCO_2 , f_{flight} , and T_{thorax} all changed significantly, but only f_{flight} was directly predicted by the asymmetry. The dynamic control of flight parameters is discussed in light of these findings, and I conclude that commonly observed intra-specific variation is meaningful and can lead to new insights into physiological control and evolution.

INTRODUCTION

The vertical integration of organismal physiology through the many levels of biology is a major contemporary challenge (Mykles *et al.* 2010). It is often difficult to link these levels because the mechanism or the extent of the variation are difficult to study. The integration of insect flight physiology is complex and dynamic, as insects simultaneously control many aspects of flight kinematics and many other aspects of metabolism and heat production. Evolutionary history can lead to significant differences between species, even among those with similar average masses inhabiting similar environments (Bartholomew & Casey 1978; Casey 1976; Darveau *et al.* 2005a; Ellington 1984; Lehmann *et al.* 2000; Niven & Scharlemann 2005). Intra-specific variation layers even more complexity on these broad patterns, and similar individuals can vary widely independently of mass (e.g., Feuerbacher *et al.* 2003; Lehmann *et al.* 2000). All else being equal, individual idiosyncrasies in relative body size parameters, such as wing planform area or the ratio of flight muscle to total mass, lead to variation in the physiology of the individual (Marden 1989; Casey 1976; Hedenström *et al.* 2001; Roberts *et al.* 2004). However, all else is not equal, and individual differences also arise from variation in behaviour and motivation (Moffatt 2001; Mapalad *et al.* 2008) or flight experience (Hesselberg & Lehmann 2009). Moreover, heat production, metabolic rate, and wing beat

frequency may covary dynamically in order to control one of the three at a desired level (Harrison *et al.* 1996b).

Body size variation strongly shapes insects' individual flight behaviours and physiology. Longer wings lead to lower wing beat frequencies in the bumblebee *Bombus vagans*, a relationship independent of other factors such as air or thoracic temperature (Joos *et al.* 1991). In *B. impatiens*, wing length and area scale positively with mass, and maximum vertical force production rapidly increases with these parameters (Buchwald & Dudley 2010). Experimental or natural wing area reduction in foraging honeybees and bumblebees impacts their foraging decisions and movements between flowers (Haas and Carter 2008; Higginson & Barnard 2004). Intra- and interspecifically in Lepidopterans, a larger body relative to wing size (higher wing loading) requires higher metabolic rates and results in higher thoracic temperatures (Casey 1976). There has been strong stabilising selection on wing morphology, shown by low natural variation in wing dimensions compared to their evolvability (*Drosophila spp.*, Houle *et al.* 2003), and decreased fitness after artificial selection for different forewing scaling allometries (*Bicyclus anynana*, Frankino *et al.* 2005). The strong impact of size on the performance of individuals explains why there has been strong stabilising selection on wing dimensions within species and groups (Frankino *et al.* 2005; Houle *et al.* 2003).

The morphological variation between species apparently coincides with significant physiological differences in parameters such as organismal and cellular metabolism or wing stroke parameters. However, it is unclear how many of these differences are due to evolved patterns, and how many arise from purely physical phenomena. One could hypothesise that all of the differences between species are simply due to a rescaling of body size.

Considering bees, larger individuals (and consequently larger species) would have larger

wings, so lower wing beat frequencies, leading to lower mass-specific metabolic rates, and in turn lower mass-specific enzyme capacities. Therefore, morphological parameters should not only correlate with flight parameters such as wing beat frequency and metabolic rate, but more importantly, the residuals of these flight parameters (with respect to body size or some other parameter) should correlate. When the residuals are correlated, this supports the hypothesis that, for example, relative changes in wing beat frequency directly causes relative changes in metabolic rate. In the absence of such correlations, one would instead suggest that, continuing the previous example, both wing beat frequency and metabolic rate are correlated only because of their mutual relationship to body size.

If the patterns within and between species are identical, this would imply that there are few evolved differences between species, and that if one species could be scaled to the dimensions of another, they would not physiologically differ. Indeed, Darveau et al. (2005a, 2005b) suggested that the correlated evolution of body size and metabolism indicates that biomechanics explains the bulk of variation between orchid bee species. Some of these inter-specific biomechanical patterns mirror those found intra-specifically (e.g., Buchwald & Dudley 2010; Joos *et al.* 1991; Roberts *et al.* 2004), but there has not, to date, been a systematic analysis of biomechanical and metabolic variation in bees. In contrast to some of the known sources of biomechanical variation, the degree to which environment and flight experience shape individual phenotypes in adult insects is essentially unknown.

Morphological patterning is static after emergence, and so has a fixed life-time effect on flight phenotypes. In order to test whether adult flight phenotypes are truly plastic, it is necessary to break any developmental correlations between morphology and physiology by manipulating either the flight apparatus (e.g., altering wing surface area) or manipulating flight experience. In this study, I explore correlated variation in morphology and flight

parameters during agitated hovering flight in bumblebee workers (*B. impatiens*), in ambient laboratory conditions. Both the bumblebees and orchid bees belong to the family Apidae. While the groups are separated by ~85 million years of evolution, the essential biomechanics of flight does not differ (e.g., asynchronous muscle). Bumblebees are large social bees and have a great range in body size within the colony, which may also be a factor that determines worker division of labour and therefore flight experience (Goulson *et al.* 2002; Jandt *et al.* 2009). Previously in this lab, Bélanger (BSc Thesis, 2009) found astonishing repeatability of flight metabolism within individuals (89% between subsequent trials) and wing beat frequency (70%). This shows that individual flight phenotypes are highly unique. I extend this work by examining how flight phenotypes are determined by a cascade of factors, including variation in size, metabolism, heat production, and wing beating. I first explore how size and flight parameters vary with respect to mass and how they covary independently of mass. I expected that I then generalise the analysis beyond body mass, to patterns of shared variation amongst all parameters, by adopting a method for interpreting covariation graphically. This leads to testable hypotheses about how variation is determined as a cascade. I attempt to test this model first by altering the flight history of some bees by restricting them from flight throughout their lifetimes, and then by clipping some bees' wings to alter power requirements by wing asymmetry. Wing asymmetry alters foraging behaviour (Haas and Cartar 2008), and so may also alter flight physiology.

METHODS

Metabolism during flight

Colony maintenance and individual marking were as previously discussed in Chapter 2. Adult workers were flown at various ages from 3 to 47 days; 35% were flown between

20-22 days of age, and 67% between 17-25 days of age. Useable flight data were obtained from 381 individuals. A daily group of 5-20 bees was collected and placed individually in 50 mL tubes. These were drilled with enough air holes so as to be essentially open, to prevent CO₂ accumulation and condensation of metabolic water which coats the bee and prevents quick stabilisation of the water signal during flight. A cotton ball with sugar-water solution was given to each bee, to standardise levels of pre-flight hunger. This minimised the probability that bees would cease flying mid-experiment, but increased the probability of metabolic water excretion mid-flight, preventing a successful water loss trace (VH₂O).

Flow-through respirometry was used to measure metabolic rate by CO₂ output, assuming that bumblebees, like other bees, fuel flight entirely with carbohydrates (Suarez *et al.* 2005). An SS3 subsampler pump (Sable Systems Int., SSI) pulled laboratory air through a drying (drierite) column, a CO₂ scrubbing (Soda Lime) column, then another drying column, at a rate of 1.5 L/min. Pressure oscillations from the pump were damped downstream in a 500 mL manifold. The airstream was then divided in half and pushed into separate channels of a FlowBar mass-flow meter (SSI), where the flow was restricted to 750 mL/min per channel. One channel was used as the reference stream, with a length of tubing matching the experimental stream, and an empty flight chamber. Individuals were flown in a 500 mL container, at ambient laboratory temperature. Incurrent air entered the base of the chamber at a right angle to the excurrent air, forcing mixing of air within the chamber. Additionally, air flowed out of the opening of the narrow jar mouth, minimising contamination of the dried and scrubbed air as the bee was placed inside. The air stream from the flight chamber passed directly to a LiCor 7000 CO₂ and H₂O gas analyser.

Baseline measurements of CO₂ and H₂O were collected prior to each flight, and each individual was flown for five minutes. The bee was encouraged to fly by vigorously shaking

the jar when it landed. Each flight was scored qualitatively based on the bee's behaviour: she mostly hovered in place or flew away from walls (high quality); she frequently hovered near the walls or floor, or landed infrequently (medium quality); she crashed or landed frequently (poor quality). Ellington *et al.* (1990) made similar distinctions based on flight quality and found differences in oxygen consumption of bumblebees. As discussed below, the poorest fliers were ultimately omitted from analyses. The highest quality flights were probably most similar to the 'uninterrupted voluntary flights' of other authors (Woods *et al.* 2005).

Wingbeat frequency in flight (f_{flight}) was obtained with an optical tachometer (Moore Scientific) and Trex 2.0 Transient Waveform Recorder software. Individuals were tracked with a direct-current light source; f is the frequency of interruption of the light source, measured in sampling bursts of ~ 0.37 s and 44.1k samples s^{-1} .

Immediately following the flight, the bee was photographed in infrared to analyse surface temperatures at the head (T_{head}), thorax (T_{thorax}), and abdomen (T_{abdomen}). Preliminary experiments indicated that surface temperature can decrease 0-2°C within 10s of terminating flight. I therefore developed a rapid restraining device by cutting the head off a 60 mL syringe, weaving fishing-line in its place. The rubber head of the piston was replaced by a cotton ball covered in aluminium foil, and the piston was used to restrain the bee. Bees were photographed within 2-3s after flight. When the bee was restrained for IR measurements with this method, its position could not be controlled, and both the size of the fishing line and the marker tag obscured some data. Two pictures were taken in succession, and if the temperature data from the first was obscured, data from the second (provided it was comparable) was substituted. Otherwise, no temperature was recorded. The resolution was

insufficient for recording average body part temperature, so I used the maximum temperature for each of the head, thorax, and abdomen.

Following IR imaging, each bee's wing beat frequency during struggle (f_{struggle}) was obtained optically, then the bee was anaesthetised with N_2 , weighed (total body mass, M_{body}), and stored at -80°C . Individuals chosen for biochemical analysis (Chapter 4) were removed from -80°C on dry ice. The thorax was dissected away on a chill plate held at -5 to -10°C , weighed (M_{thorax}), and replaced at -80°C . Wings were pasted onto white paper, scanned, and analysed with ImageJ software for forewing and hindwing surface area (SA_{FW} and SA_{HW} , and sum to $\text{SA}_{\text{planform}}$).

Manipulation of bee flight history

The influence of flight behaviour on physiology was examined by manipulating bees' flying experience. Three colonies were raised in the unrestricted treatment, by placing the colony box in a $0.6\text{m}\cdot 0.6\text{m}\cdot 1.8\text{m}$ mesh enclosure and three colonies were raised in the restricted treatment, inside their boxes in darkness to minimise or prevent flight. Three to four times daily, each colony was shaken vigorously, inducing guarding flights lasting ~ 1 - 4 minutes. The restricted colonies were also shaken to standardise the effect of stress alone, and when observed with infrared imaging, bees in the dark only buzzed in response to the shaking stimulus, and did not fly. Once per day, a dummy box with a one-way entrance was substituted into the unrestricted bees' enclosure while they were performing guarding sorties, and the bees collected inside as they returned. I recorded the IDs of the collected bees, after which they were returned to their enclosure and original colony.

A subset of individuals from one unrestricted colony was further manipulated by introducing wing surface area asymmetry. Wing asymmetry results in larger changes in flight behaviour than symmetrical surface area reduction (Haas and Cartar 2008), and so I expected it to be a greater physiological challenge. After marking of new young adults, the outer trailing edge of the left forewing of a new worker was cut with fine scissors (Hedenström *et al.* 2001). The portion of cut wing was treated as a continuous range from 15-40% asymmetry.

Data analysis

Data analysis was performed in the R language environment (v 2.10, R Development Core Team 2009). Restricted and unrestricted bees were initially treated together, and flight quality and colony of origin included as covariates in all analyses. Comparisons of the effect of restriction were made with respect to flight quality and body mass, but since wing cutting was performed only within a single unrestricted colony, so only flight quality was included as a covariate. Sequential linear models (type I sums of squares) were used to examine the statistical significance of single terms accounting for covariation with continuous or categorical variables. Type II sums of squares were used when the significance of two or more terms was being studied. Partial F-statistics are reported with degrees of freedom in subscript, and where included, R^2 are partial with respect to the other terms in the model (R_m^2 , where m is the number of independent model variables).

I first examined flight parameters with respect to mass, to compare scaling exponents (M^a) with respect to body mass (M_{body}) or thorax mass (M_{thorax}). Exponents (log-log regression coefficient) and R^2 (partial sum of squares (SS) / total SS) were estimated in 1000 subsamples of 4/5 of the data. The reported values are the mean and standard deviation (sd)

of the subsamples. The relationship was considered to be significant if at least 950/1000 subsamples were significant.

Variation in flight parameters is not solely due to body size, and so correlated variation in the parameters was investigated while controlling for M_{body} as a covariate. This method examines how departures from the predicted body size relationship in one variable account for similar departures in another variable. Each parameter can be correlated with mass, but residual variation remains in the regression. This residual variation can be extracted as a new variable, and correlated with other measures, which is the basis of residuals analysis (*e.g.*, Joos *et al.* 1999; Rezende *et al.* 2009). Rather than creating a new variable, I report the partial correlation between the two parameters, given covariation with another parameter (Zar 1999). Visually, the question is presented as, if a variable falls below the regression line (for instance, a smaller wing area than expected by body size), do related parameters also fall below the line (such as wing beat frequency)? All statistical results are reported as F-statistics with degrees of freedom in subscript and p-value.

Although covariation with mass may significantly explain many parameters, there can also be covariance amongst the parameters themselves. The previous method for investigating the partial correlations after accounting for mass was generalised to an automated procedure for examining the partial correlation with respect to every other parameter, in sequence. For instance, the correlation between $V\text{CO}_2$ and f_{flight} is tested for significance after considering possible covariation with every other variable in the study. The relationship is considered significant if and only if there is no alternative hypothesis explaining the variation between these two (such as covariation with body temperature or wing surface area).

The relationships that are significant based on the above approach approximate an undirected dependency graph (UDG; de la Fuente *et al.* 2004; Shipley 2002). The directionality of relationships is not tested in the UDG, so it avoids common pitfalls in path analysis (Petraitis *et al.* 1996; Shipley 2002). In principle, the significance of the partial correlation between any two variables should be tested after taking into account all other variables, since the maximum path length between any pair of variables in a connected network (no orphans) is $n-2$, where n is the number of nodes. However, de la Fuente *et al.* (2004) found that accounting for just the second order partial correlation in a much larger metabolomic data set was sufficient (they also discuss why limiting the number of simultaneously considered variables may be important).

Using the UDG as a guide to the most significant paths, the proportion of explained variation for each focal variable was inferred from the adjusted R^2 of a statistical model composed of all the variables linked directly to the focal variable. However, morphological variables were only predicted from each other, and not from flight parameters. This proportion of explained variance is overlaid on the UDG.

RESULTS

Variation in body size and flight parameters

Workers in the six colonies ranged in mass from 50-250 mg. M_{body} was tested first and showed large differences between colonies (Table 3.1). M_{thorax} differed between colonies, independently of mass, so the ratio $M_{\text{thorax}}/M_{\text{body}}$ differed among colonies. Wing area parameters differed between colonies only with respect to the changes in M_{thorax} , and there were no significant interactions between mass and colony. Moreover, when both M_{thorax} and M_{body} were included together in a model to predict wing parameters, only M_{thorax}

was significant. I examined the scaling of size measures with M_{body} and M_{thorax} to examine the consistency of individual body plans (Table 3.2). In all cases, the exponents predict allometry, so larger individuals are designed along slightly different proportions than small individuals. M_{body} explained 55-71% of variation in other size parameters. M_{thorax} explained more variance, but even so only about 70-85% of the variation in wing morphology. SA_{HW} was better predicted than SA_{FW} , and also increased with body size more quickly.

Flight parameters varied enormously within a body size range (Fig. 3.1). For instance, at 140 mg, VCO_2 ranged from 6-12 mL CO_2 hr^{-1} , f_{flight} from 170-230Hz, and T_{thorax} from 35-41°C. Some of this variation is influenced by colony of origin and flight quality. The poorest fliers (black circles, Fig. 3.1), had very low metabolic rates and significantly different slopes on M_{body} in some cases, and so were removed from all subsequent comparisons. Flight quality was generally a significant main effect (Table 3.3). There was a small, though significant, interaction between M_{body} and flight quality ($M_{\text{body}} \times$ Flight quality) when predicting flight wing beat frequency (f_{flight}). $M_{\text{body}} \times$ Colony was significant only in predicting T_{head} . Because the two interaction terms were of weak significance, accounting for them did not result in qualitative changes to the results, and subsequently only the main effects were considered. Variation in flight parameters was poorly predicted by either measure of mass, explaining at most ~40% of variation. VCO_2 and body temperatures were better explained by, and scaled more steeply with, variation in M_{body} than with M_{thorax} . T_{abdomen} was not at all predicted from M_{thorax} . VH_2O and both wing beat frequencies were substantially better predicted by M_{thorax} than M_{body} , and f_{struggle} was not related to M_{body} .

Body mass-independent variation

Body mass is an important primary determinant of flight parameters, so mass-independent correlations were examined for additional sources of covariation among flight parameters. I first examined how variation in relative size proportions might affect flight performance. When either of the total wing surface area (SA_{planform}) or M_{thorax} was smaller than expected for a given value of M_{body} , f_{flight} was greater (SA_{planform} in Fig. 3.2a; SA_{planform} : $F_{1,116}=97.05$, $p<0.001$; M_{thorax} : $F_{1,107}=34.47$, $p<0.001$). But M_{thorax} and SA_{planform} are intrinsically correlated (Table 3.1), so I examined them simultaneously to account for their covariation. Together, they both significantly predicted f_{flight} (M_{thorax} : $F_{1,99}=7.82$, $R_4^2=0.03$; SA_{planform} : $F_{1,99}=43.31$, $R_5^2=0.14$; M_{body} excluded as nonsignificant in this model). Surprisingly, the contribution of M_{thorax} to f_{flight} was either zero or slightly positive when it was considered together with SA_{planform} (Fig. 3.2b). Changes in VCO_2 with respect to M_{thorax} and SA_{planform} were explained by covariation with f_{flight} and M_{body} (SA_{planform} : $F_{1,97}=2.82$, $p=0.09$; M_{thorax} : $F_{1,97}=0.43$, $p=0.51$, f_{flight} : $F_{1,97}=56.69$, $p<0.001$).

Relative variation in one flight parameter with respect to size can have consequences for other parameters. Although the correlation of absolute VCO_2 and f_{flight} was significant ($F_{1,331}=7.00$, $p=0.009$), it was extremely poor ($R_3^2=0.003$; Fig. 3.3a). Conversely, when f_{flight} was higher than expected for a given mass, so was VCO_2 ($F_{1,282}=95.57$, $p<0.001$, $R_4^2=0.09$; Fig. 3.3b). However, both covaried with thorax temperature (T_{thorax}), and taking this into account resulted in a weaker association again ($F_{1,251}=21.94$, $p<0.001$, $R_5^2=0.01$; Fig. 3.3c). This shows that correlations with body size can be mediated by another physiological parameter. T_{thorax} increased with M_{body} but so did mechanisms of heat production, like VCO_2 and f_{flight} (Fig. 3.1). M_{body} -independent T_{thorax} correlated nearly equally with VCO_2 and f_{flight} (Figs. 3.4a and b; VCO_2 : $F_{1,254}=190.60$, $p<0.001$, $R_3^2=0.24$; f_{flight} : $F_{1,252}=189.40$, $p<0.001$,

$R_3^2=0.24$). Accounting for these sources, the impact of M_{body} on T_{thorax} was reduced from $R_3^2=0.13$ to $R_5^2=0.01$ (though still significant, $F_{1,248}=7.13$, $p=0.01$; Fig. 3.4c).

Undirected dependencies of flight parameters

The previous analyses show the importance of covariation in measuring the apparent impact of one variable on another. Using a graph where nodes are variables and edges are significant pairwise correlations, all the significant direct (Fig. 3.5a) and M_{body} -independent correlations (Fig. 3.5b) can be summarised. This shows that accounting for covariation removes many pairs of significant relationships. Nonetheless, there are significant remaining sources of covariation, such as VCO_2 , f_{flight} , and T_{thorax} . The method of examining M_{body} -independent partial correlations between variables was generalised to the partial correlations with respect to every other variable in the study. The partial correlations can then be used to approximate the undirected dependency graph (UDG) of relationships between body size and flight variables. Many indirect paths are filtered with this method, though the graph is still connected (Fig. 3.6a), and variables group according to function: body size, metabolism, body temperature, and wing stroke.

The partial correlations discriminate between best size predictors of flight parameters, and probable indirect paths from size to other variables. M_{body} was a better predictor of VCO_2 than were other measures of size; in turn, VCO_2 predicted VH_2O . Conversely, f_{flight} was predicted by a combination of SA_{planform} and M_{thorax} . Despite the better prediction of f_{struggle} by M_{thorax} than M_{body} (Table 3.1), size dimensions did not predict f_{struggle} more than expected by f_{flight} . As suggested previously, M_{body} only predicted thoracic temperatures (T_{thorax}) indirectly through VCO_2 and f_{flight} ; in turn, variation in T_{thorax} predicted variation in T_{head} and T_{abdomen} .

M_{body} -independent variation can again be layered on this, by holding M_{body} as a constant covariate while cycling through other parameters (Fig. 3.6b). The same relationships hold, except for the mass-independent appearance of the correlation between VCO_2 and f_{flight} , and the removal of the link between f_{flight} and M_{thorax} . The latter suggests, in conjunction with the weak R^2 previously, that this link is not very robust.

Manipulated Flight History

I manipulated individuals' flight histories to examine whether changes occurred in correlated variables, and if these were predicted by the paths. Flights were provoked by agitating the colony; insofar as the first recorded daily observation of an individual reflects its first flight, 55% of bees made their first flights within 2-5d post-emergence. Bees in the restricted treatment were ~10% larger, so M_{body} was used as a covariate for all comparisons. Results are shown in Fig. 3.7. Restricted bees had reduced VCO_2 and T_{thorax} (VCO_2 : $F_{1,300}=29.81$, $p=0.002$; T_{thorax} : $F_{1,261}=16.67$, $p<0.001$), but no change in f_{flight} ($F_{1,293}=1.57$, $p=0.21$). The effect size was small, which might have been because within the unrestricted treatment, many more individuals flew rarely than often (Fig. 3.8a). I then examined the impact of number of lifetime recorded flights, to see how this might affect the results. Within the unrestricted treatment, only VCO_2 increased with the number of flights (Fig. 3.8b, $F_{1,123}=21.91$, $p<0.001$).

In spite of this, the paths in Fig. 3.6a predicted that covariation amongst parameters should be taken into account to examine overall differences between treatments. Rather than comparing bees based on flight restriction or not, bees were tested for the continuous effect of lifetime flights. Bees in the restricted treatment were assigned zero lifetime flights (Table 3.4). Lifetime flights strongly impacted VCO_2 , but only slightly affected f_{flight} . The effect of

flight experience on T_{thorax} was accounted for by $V\text{CO}_2$ and f_{flight} . In this model, the impact of flight quality was most evident on T_{thorax} . Lifetime flights could represent damage or natural ageing of muscle coupling (e.g., wear or oxidation). However, the number of flights in the present study was no more than 2% of the greatest number of lifetime foraging trips in a similar indoor enclosure (Levente Orbán and Skandalis, unpub. data), flights which were also of much longer duration. Also, age did not affect $V\text{CO}_2$ overall or in either treatment alone (overall: $F_{1,249}=1.54$, $p=0.22$), nor was the effect of lifetime flights explained by age as a covariate (age: $F_{1,95}=0.69$, $p=0.42$).

Asymmetric Wing Area

I then challenged bees in one unrestricted colony by reducing wing surface area, and compared the effect of wing cutting on individuals from the same colony. The effect of asymmetry was not continuous (Fig. 3.9), so asymmetry's effect on $V\text{CO}_2$, f_{flight} , and T_{thorax} was examined as its presence or absence (Table 3.5). $V\text{CO}_2$ was predicted by flight number, but not wing asymmetry. Conversely, f_{flight} was related to wing asymmetry, but otherwise not to flight number. Both were related to T_{thorax} , which, again, was not related to M_{body} .

DISCUSSION

I examined inter-individual differences in flight behaviour in order to assess how flight phenotypes vary across individuals. Testing variation in phenotypes requires that individuals vary uniquely. Bélanger (2009) tested this by measuring repeatability of individuals' performance, and found that depending on the measure, 70-90% of the variance in subsequent tests on the same individual was shared between trials. Since she did not control for flight quality (unless that, too, is repeatable), this is likely a conservative estimate of repeatability.

Data can also be compared for similarity in scaling and variability; values from the literature were obtained and compared in Table 3.2. Examples in *Bombus* include Joos *et al.* (1991), who found M_{thorax} was 90-97% explained by M_{body} , while Buchwald and Dudley (2010) found that muscle mass (M_{muscle} , ~89% of M_{thorax}) was perfectly predicted by M_{body} ($R^2 \sim 1.00$). For the flight parameters, Bélanger (2009), found that M_{body} explained 60% of the variance in VCO_2 , whereas I found only 37%; she also found 17% of the variance in f_{flight} explained by M_{body} , while I found about 9%. Besides the fact that R^2 vary with sample size (lower at higher n), there may be some biological reasons for the different proportions of explained variation. I offered bees nectar prior to testing, so some ate and some did not (standardised hunger rather than feeding). This might have increased the variability of M_{body} , but if this is the reason, it reflects positively on the results. Since I have concentrated on correlated variability, an increase in the diversity of M_{body} with respect to size and flight parameters increases the resolution of the analysis. Another possibility is that there were differences in the selection of data, since I forced all individuals to fly, by vigorous encouragement if necessary. This was partly accounted for by recording flight quality: excluding it as a covariate increased R^2 by as much as 10% or more. But qualitatively scoring flight is not equivalent to subselecting the best fliers based on motivation and behaviour. In my experiment, behavioural differences led to more variability with respect to size, which might have biased predictions of covariation toward flight parameters. The interpretability of results based on different handling and flight methods has been frequently criticised (e.g., Woods *et al.* 2005), so cautiously the UDG of Figs. 3.6 could be caused by behavioural variation. Nonetheless, as discussed in the Introduction, the paths in both panels of Fig. 3.6 provide a reference for comparison of covariation in flight parameters in different environments or even flight conditions.

In general though, it seems inappropriate to discount covariation among flight parameters, or apply single-causes explanations, such as body mass, to a dynamic system (the argument of Darveau *et al.* 2002). Testing of the partial correlations between pairs of variables with respect to all other variables resulted in intuitively valid biological relationships, all of which reflect the same organisational principles as found by Darveau *et al.* (2005a) in orchid bees. Interestingly, physiological functional groups can be identified based on their adjacency in the graph: body size, metabolism, thermoregulation, and wing stroke parameters. In my subsequent manipulations, I found that the impact of experimental treatments was best understood when variation in each of these functional groups was considered simultaneously.

Based on the static morphology of metamorphosed adults, I suggest that much of the individual variation in flight develops from idiosyncrasies in body size patterning. The lower predictability of total mass in adults, compared to other measures of morphology, reflects changes in abdominal mass through feeding, depletion of energy stores, ovarian development, etc. Dimensions of the thorax, i.e., M_{thorax} and SA_{planform} , are more closely related because they are developmentally linked. The result is that different measures of body size are not necessarily equivalent (Skandalis *et al.* 2009), and so affect flight performance differently (Fig. 3.6). VCO_2 is best predicted by M_{body} , as the flying bee must generate enough force to lift its own weight. Relatively smaller wings required higher f_{flight} in bees of the same mass (Fig. 3.2a), which then required greater VCO_2 . The prediction of VH_2O solely from VCO_2 suggests that water loss is primarily metabolic. This is corroborated by the apparent VH_2O outlier at 170 mg (Fig. 3.1b), which actually had a very high mass-independent VCO_2 .

The independent contributions of different, though functionally linked, size parameters has been commented on in the past (Srygley & Kingsolver 1998). Many studies have examined the effect of M_{body} on flight performance, either naturally or experimentally with added weights, and drawn the conclusion that the ratio $M_{\text{muscle}}/M_{\text{body}}$ is of prime importance (Almbro & Kullberg 2008; Marden 1989; Roberts *et al.* 2004; Srygley & Kingsolver 2000; Vance *et al.* 2009). However, it is difficult to discriminate between the ratios $M_{\text{muscle}}/M_{\text{total}}$ or $SA_{\text{planform}}/M_{\text{body}}$ when only M_{body} changes. After taking into account SA_{planform} , I found that M_{thorax} or M_{body} positively predicted f_{flight} , if it predicted f_{flight} at all (Fig. 3.2b). Instead, this suggests larger individuals have lower f_{flight} because their wings are larger (e.g., Joos *et al.* 1991). In a species of butterfly with males and females of nearly identical $M_{\text{muscle}}/M_{\text{body}}$, the males have lower wing-loading and increased flight performance characteristics (daily flight distance; Mendoza-Cuenca & Macías-Ordóñez 2005). Experimental reduction of wing surface area in dragonflies impairs aerial performance, though muscle mass is unchanged (Combes *et al.* 2010). Growth of *D. melanogaster* in cold environments leads to altered flight performance ascribed primarily to differential growth of the wings (Frazier *et al.* 2005). This highlights the need to account for covariation in developmentally and functionally linked size parameters.

Though wing area scaled with M_{body} (Table 3.2), SA_{HW} scaled faster than SA_{FW} , and these differential scaling rates might have functional consequences. Despite being structurally linked to the forewing, Buchwald & Dudley (2010) found that removing the hindwing causes a startling decrease in f_{flight} . This may indicate a role for the hindwing in activating the wing stroke, since wing size reduction is expected to cause an increase in f_{flight} (Hedenström *et al.* 2001; Jantzen & Eisner 2008). Similar, though less pronounced, decreases in f_{flight} were found with different combinations of fore- and hindwing clipping in

locusts (*Schistocerca gregaria*), although their wings are not coupled (Fischer & Kutsch 2000). With larger wing areas, the comparatively larger SA_{HW} may help control flight through feedback from force generation. In an attempt to dissociate the component of f_{flight} due to control of flight forces, I conceived of measuring $f_{struggle}$ while the bee is grasped between forceps. I expected this to correlate exclusively with wing and thorax dimensions, which would imply that $f_{struggle}$ is the frequency due solely to the coupling of muscle contractions to the wing apparatus. $f_{struggle}$ is indeed lower than f_{flight} by $\sim 10\text{Hz}$, and unlike the latter, is not at all predicted by M_{body} (Table 3.2). Accordingly, I expected that $f_{struggle}$ would pose as a link from body size to f_{flight} , though this was not the case (Fig. 3.6). $f_{struggle}$ is not as well predicted, and there may be several behavioural and physical reasons for this. In particular, $f_{struggle}$ tended to decrease over time, so estimating its mean was difficult. Thus the data do not support a clear meaning or utility of this variable, even though it differs between individuals.

The strong impact of morphology on flight performance is the vertical element of the cascade of physiological contributions, because it is mostly invariant in the adult. The second element is lateral contributions between the flight parameters themselves. I found flight parameters were highly correlated with each other (Fig. 3.6), and several, such as V_{H_2O} , T_{head} and $T_{abdomen}$, were predicted only as indirect consequences of metabolism. Sensibly, f_{flight} and V_{CO_2} ought to be linked because power generation must be met with metabolic supply (Lehmann 2001), but there is at best a small correlation between V_{CO_2} and f_{flight} either absolutely or after taking into account covariation with T_{thorax} . One might interpret the low R^2 to mean their association is not very meaningful, and this certainly seems to be true in absolute terms. However, one interpretation of Fig. 3.4c, is that in spite of the important influences of M_{body} and T_{thorax} , an individual with a higher f_{flight} still must have a higher

VCO_2 . This raises the question of whether T_{thorax} should even be treated as a covariate. A case can be made that T_{thorax} is itself an indirect calorimetric experiment, and so partly redundant to VCO_2 , with differences reflecting constraints of the respective methods. In that case, the strong correlation of f_{flight} and VCO_2 indicates a very strong coupling of energetic demand and supply.

Despite this, uncoupling of VCO_2 and f_{flight} has been observed frequently. Insects can accomplish broad changes in lift generation independently of increases in metabolic power by compensatory adjustments to the wing stroke (Hedenström *et al.* 2001; Lehmann & Dickinson 1997). Increased metabolic output can also be uncoupled from changes in the wing stroke, as occurs in honeybees lifting loads (Feuerbacher *et al.* 2003). I suggest that direct comparisons of metabolic demand and supply may not be considering the important inter-individual contributions of thermoregulation; multiple effects of temperature make it simplistic to require that VCO_2 , T_{thorax} , and f_{flight} interact additively. For instance, VCO_2 and f_{flight} contribute directly to elevated T_{thorax} (Fig. 3.6). But bees flying at high operating temperatures achieve optimal conditions for wing beating (Coelho 1991) and metabolic efficiency through temperature effects on muscle biophysics (Gilmour & Ellington 1993). So more likely, control of flight parameters is an implicit optimisation by each individual, and includes feedback between parameters. Further study of this problem will require better estimation of power output, since f_{flight} is just one component of mechanical power output, which is ultimately the product of f_{flight} and stroke amplitude (Lehmann & Dickinson 1997). Measuring temperature at the surface of a sphere may also cause bias over a large range of body sizes, because of heat dissipation over different spheres' surface areas to volume.

Other considerations than metabolic efficiency may influence the regulation of core flight parameters. Bees regulate T_{head} , $T_{abdomen}$, and T_{thorax} with different slopes on

temperature (Harrison *et al.* 1996b; Woods *et al.* 2005), but at a single temperature, I have found that temperature is primarily related to T_{thorax} , where the heat is produced. Bees might have reason and means to attempt to regulate T_{head} and T_{abdomen} independently of T_{thorax} . Inter-individual differences in T_{head} could lead to variability in perception and behaviour. Elevating T_{head} through T_{thorax} may positively contribute to neural processing (to a point), and might be a motivation for heating up during high-reward foraging (Moffatt 2001; Mapalad *et al.* 2008). T_{head} was substantially better predicted than T_{abdomen} (Fig. 3.6), and this might have been because, despite haemolymph flow, bumblebees restrict heat loss to the abdomen by counter-current exchange at the petiole; at higher temperatures, they circumvent this mechanism to shunt more heat from the thorax (Heinrich 1976). The lower predictability of T_{abdomen} might also be because the thorax heats rapidly, but the abdomen may take many minutes to reach its peak temperature, possibly longer than the flight assays (Stone 1993; though there was no change in T_{abdomen} with flight duration in honeybees, Roberts & Harrison 1999).

These many considerations of various impacts arise because biologically and statistically, the directions of the paths in Fig. 3.6 influence how the correlations are performed and our understanding of causation within the network. Properly, this is the domain of structural equation modelling (Shipley 2002). I attempted to empirically evaluate the paths by manipulating flight history to observe correlated changes. If one parameter, such as VCO_2 , can change independently of T_{thorax} and f_{flight} , this would challenge the model of a cascade. Both experiments support the conclusion that variation in heat production is related only to variation in f_{flight} and VCO_2 . On the other hand, the lack of a profound correlation between f_{flight} and VCO_2 among individuals or the lack of correlation at all in the wing cutting experiment, would suggest an alternate path, that they are somehow linked by

T_{thorax} (though in the experiment, the resolution of relationships with low R^2 is reduced by the smaller sample size).

The results of the experiments contrasted depending on whether flight parameters were considered individually (Fig. 3.7 and 3.9), or simultaneously with others (Tables 3.4, 3.5). This is a natural consequence of the connectedness of the physiological network (Fig. 3.6). Taking into account correlated changes in VCO_2 and T_{thorax} , there was a small effect of lifetime flights on f_{flight} , which was not observed directly. While wing asymmetry did impact f_{flight} , the directly observed increase in VCO_2 (Fig. 3.9a) was not predicted either by the asymmetry or increased f_{flight} . Instead, the analysis suggests it appeared indirectly with elevated T_{thorax} and lifetime flights.

The experiments touch on the roles of integration of the flight system on the natural ecology of insects. Honeybees quickly gain flight experience and increase their foraging efficiency (Schippers *et al.* 2006). In this study, flight experience also resulted in increased VCO_2 and T_{thorax} (Fig. 3.7a and c; Table 3.4), which is in the same direction as high-reward foraging (Mapaldi *et al.* 2008; Moffatt 2001). Inexperienced fliers of *D. melanogaster* largely show reduced flight control rather than altered kinematics (no change in f_{flight} or stroke amplitude; Hesselberg & Lehman 2009). In this study, differences in f_{flight} were only found after accounting for substantial covariation.

Hedenström *et al.* (2001) suggested that symmetric wing damage may affect survival of bumblebees mostly through reduced maneuverability, rather than by imposing an energetic cost. Complete removal of the hindwings of Lepidoptera (-50% SA_{planform}) had limited impact on f_{flight} (or slightly positive) but resulted in a strong reduction in aerial maneuvering, and so predator avoidance (Jantzen & Eisner 2008). Predators are similarly

constrained, and wing damage reduced aerial performance and hunting ability (Combes *et al.* 2010).

However, honeybees with wing damage appear less stringent in their cost-benefit analysis of foraging, and opt for closer, though less rewarding, forage (Higginson & Barnard 2004). This could imply an energetic constraint. I found that the elevated cost of flight (VCO_2) with asymmetry (Fig. 3.9a) was best explained indirectly through higher T_{thorax} (Table 3.5), which itself was probably due to the elevated f_{flight} . This differs from Hedenström *et al.* (2001), who did not find a change in metabolic rate, but I tested up to to 40% wing reductions, compared to their ~10%. Cartar (1992) observed that the natural extent of wing wear of bumblebees in the field is typically not in excess of ~20%; at that level of asymmetry, I did not find consistent differences in any of VCO_2 , f_{flight} , or T_{thorax} (Fig. 3.9). While bumblebee flight behaviour is generally robust to wing damage, higher asymmetry and wing loading result in more erratic flight paths (Haas and Cartar 2008). So while providing some experimental evidence in support of Fig. 3.6, the results also support reduced maneuverability as the greater cost to individuals, rather than energetics. That said, the location and manner of damage may be of critical importance. Both Buchwald and Dudley (2010) and Fischer and Kutsch (2000), actually found reductions in f_{flight} when the hindwing was removed, which is opposite to expectation. The reduced f_{flight} would be a strong test of the paths in Fig. 3.6, and should result in lower VCO_2 and T_{thorax} .

In conclusion, using methods to compare sources of correlated inter-individual variation, I suggest that unique physiological phenotypes can, and should, be understood as a cascade of interacting components. A graph theoretic approach was adopted to model this variation in bumblebee workers, and also suggests the possible study of feedback and nonlinear control over flight parameters. Relative variation and idiosyncrasies in bee size

directly predict variation in a few core components such as VCO_2 and f_{flight} , which in turn predict other factors such as T_{thorax} . This reflects both vertical and lateral integration of flight parameters. These patterns are the same observed inter-specifically in orchid bees and other insects (Darveau et al. 2005a), and so likely reflect general biomechanical rules governing flight. The graphical model of integration suggests potential ecological consequences of inter-individual variability, as well as reflecting why there has been stabilising selection on consistent scaling and morphological parameters (Frankino *et al.* 2005). The method of partial correlations generalises residuals analysis, but rather than examining residuals with respect to just one variable, such as mass (e.g., Rezende *et al.* 2009), it is possible to extract covariation from all other variables simultaneously. This process can recapitulate experimentally demonstrated relationships, clarify some hypotheses, and fuel controversy in others.

Table 3.1 Body mass and colony effects on parameters of body size parameters. Model independent variables are listed by rows, and dependent variables in columns. Linear model were tested by type II sums of squares. F-statistics are provided with degrees of freedom in subscript where the term is significant. All bolded terms are highly significant ($p < 0.001$).

Predictor	Dependent				
	M_{body}	M_{thorax}	SA_{planform}	SA_{FW}	SA_{HW}
M_{body}	-	F_{1,137}=580.28	ns	ns	ns
M_{thorax}	-	-	F_{1,103}=337.59	F_{1,103}=251.54	F_{1,103}=370.33
Colony	F_{5,339}=20.60	F_{7,134}=10.84	ns	ns	ns
M_{body} x Colony	-	ns	ns	ns	ns

Table 3.2 Mean values and mass-scaling scaling exponents of for size and flight parameters. Scaling exponents, a , were calculated accounting for intercolony variation (for body size measures and $f_{struggle}$) or additionally with respect to flight quality (metabolism, f_{flight} , and body temperatures). Means \pm standard deviations (sd) were not adjusted (sample size, n , in parentheses). The scaling exponent is provided as the range obtained from the mean \pm sd of 1000 subsamples of 4/5 of the data. Insignificant relationships are not reported (ns). To contrast, literature scaling coefficients with respect to M_{body} and reported R^2 are provided.

	Mean \pm sd (n)	Scaling exponent, a		Literature
		$M_{body}^a (R^2)$	$M_{thorax}^a (R^2)$	$M_{body}^a (R^2)$
M_{body} (mg)	140.0 \pm 36.8 (312)			M_{body} : 100-372 mg ¹
M_{thorax} (mg)	50.34 \pm 11.8 (133)	0.91-0.95 (0.65 – 0.71)		1.00-1.06 (0.90-0.97) ⁴
$SA_{planform}$ (mm ²)	69.1 \pm 12.8 (144)	0.66 – 0.70 (0.57 – 0.63)	0.73 – 0.75 (0.74 – 0.80)	0.59 – 0.76 (0.89) ¹
SA_{FW} (mm ²)	48.5 \pm 8.5 (144)	0.63 – 0.67 (0.55 – 0.61)	0.70 – 0.72 (0.71 – 0.77)	
SA_{HW} (mm ²)	20.6 \pm 4.1 (144)	0.73 – 0.77 (0.61 – 0.67)	0.80 – 0.82 (0.78 – 0.84)	
VCO_2 (mL CO ₂ hr ⁻¹)	9.7 \pm 2.6 (325)	0.71 – 0.77 (0.34 – 0.40)	0.59 – 0.69 (0.25 – 0.37)	0.98 (0.60) ²
VH_2O (Pa H ₂ O hr ⁻¹)	0.99 \pm 0.38 (161)	0.97 – 1.07 (0.27 – 0.33)	1.08 – 1.30 (0.29 – 0.41)	
f_{flight} (Hz)	206.1 \pm 18.9 (328)	-0.13 – -0.11 (0.07 – 0.11)	-0.22 – -0.20 (0.31 – 0.37)	-0.27 – -0.13 (0.57) ¹ -0.30 (0.45) ² -0.09 (0.17) ³
$f_{struggle}$ (Hz)	193.5 \pm 17.5 (315)	ns	-0.17 – -0.13 (0.13 – 0.18)	
T_{thorax} (°C)	37.5 \pm 1.7 (267)	0.07 – 0.09 (0.13 – 0.17)	0.05 – 0.07 (0.06 – 0.10)	
T_{head} (°C)	33.9 \pm 1.6 (261)	0.07 – 0.09 (0.12 – 0.16)	0.05 – 0.06 (0.06 – 0.10)	
$T_{abdomen}$ (°C)	30.2 \pm 1.7 (268)	0.04 – 0.06 (0.03 – 0.05)	ns	

¹Buchwald & Dudley (2010): *Bombus impatiens*; ²Belanger (2009): *B. impatiens*; ³Hedenström *et al.* (2001): *B. terrestris*; ⁴Joos *et al.* 1991.

Table 3.3 Main and interaction effects on flight parameters. Model independent variables are listed by rows, and dependent variables in columns (with sample size, n). Linear model were tested by type II sums of squares. The worst quality fliers were omitted when testing effects on f_{flight} . F-statistics are provided with degrees of freedom in subscript where the term is significant. Highly significant terms ($p < 0.001$) are bolded; otherwise, an asterisk (*) indicates $p < 0.05$.

Predictor	Dependent					
	VCO ₂ ($n=328$)	VH ₂ O ($n=192$)	f_{flight} ($n=284$)	T _{thorax} ($n=255$)	T _{head} ($n=249$)	T _{abdomen} ($n=256$)
M_{body}	F₁=308.67	F₁=105.85	F₁=37.22	F₁=53.28	F₁=89.81	F₁=10.44
Flight quality	F₂=49.86	ns	F₂=12.66	F₂=24.40	F₂=9.70	ns
Colony	F₅=3.58	ns	F₅=5.01	F₄=5.52	F₄=5.32	ns
M_{body} x Flight quality	ns	ns	F ₂ =2.88*	ns	ns	ns
M_{body} x Colony	ns	ns	ns	ns	F ₄ =3.94*	ns

Table 3.4 Effects of flight restriction on flight phenotypes. Model independent variables are listed by rows, and dependent variables in columns. Linear model were tested by type II sums of squares. $n=251$ individuals. Highly significant terms ($p<0.001$) are bolded; otherwise, * indicates significant at $p<0.05$, or ns indicates not significant.

	VCO₂	<i>f</i>_{flight}	T_{thorax}
M_{body}	F₁=213.83	F₁=152.47	ns
VCO₂	-	F₁=12.19	F₁=56.20
<i>f</i>_{flight}	F₁=20.53	-	F₁=59.01
T_{thorax}	F₁=59.01	F₁=56.20	-
Flight number	F₁=22.71	F ₁ =5.68*	ns
Flight quality	F ₁ =5.09*	F ₁ =5.15*	F₁=20.19

Table 3.5 Effects of wing cutting on flight phenotypes. Model independent variables are listed by rows, and dependent variables in columns. Linear model were tested by type II sums of squares. $n=53$ individuals. Highly significant terms ($p<0.001$) are bolded; otherwise, * indicates significant at $p<0.05$, or ns indicates not significant.

	VCO₂	<i>f</i> _{flight}	T_{thorax}
M_{body}	F₁=58.66	F₁=22.63	ns
VCO₂	-	ns	F₁=14.42
<i>f</i> _{flight}	ns	-	F₁=26.97
T_{thorax}	F₁=14.42	F₁=26.97	-
Flight number	F ₁ =8.31*	ns	ns
Asymmetry present	ns	F ₁ =6.46*	ns
Flight quality	ns	ns	F ₁ =4.13*

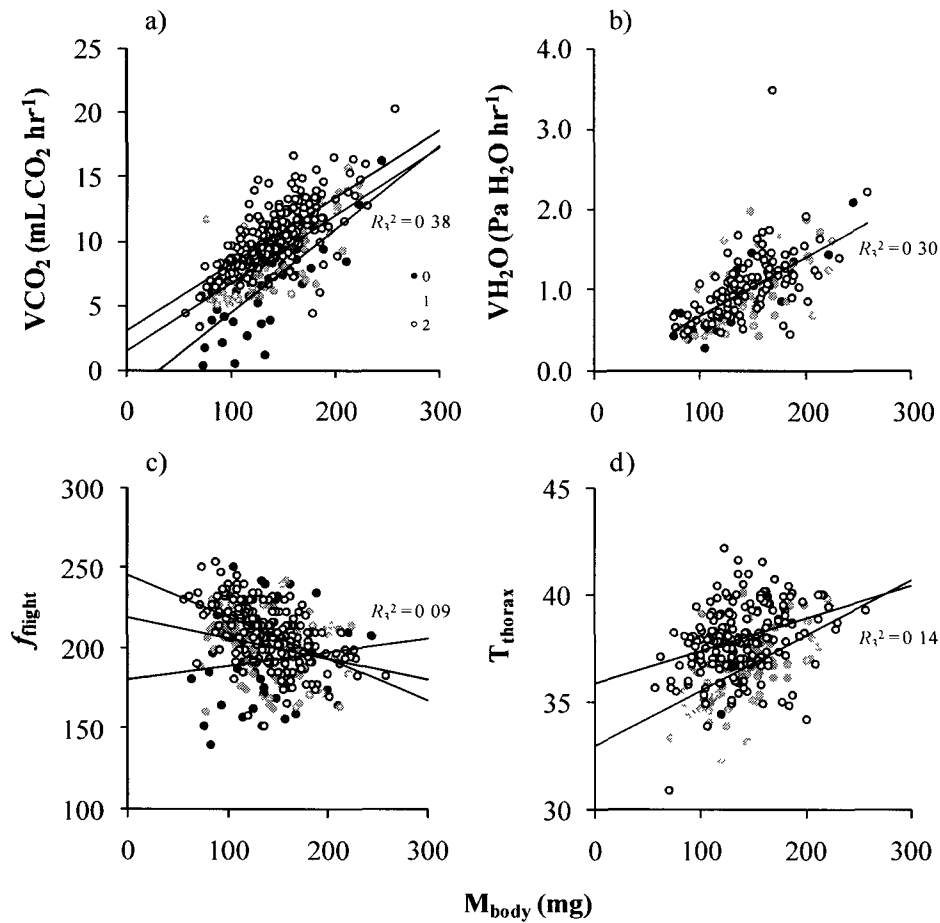


Figure 3.1 Correlation of total body mass (M_{body}) to flight metabolic rate ($V\text{CO}_2$, panel a), water loss rate ($V\text{H}_2\text{O}$, panel b), wing beat frequency (f_{flight} , panel c), and thorax temperature (T_{thorax} , panel d). Data are coded according to a qualitative scoring of flight: poor (0) to best (2), though the poorest fliers were omitted from all analyses. More than one regression lines is shown where flight quality leads to significant differences in the intercept, but I report the partial R^2 (R_p^2) of M_{body} after taking into account flight quality and colony.

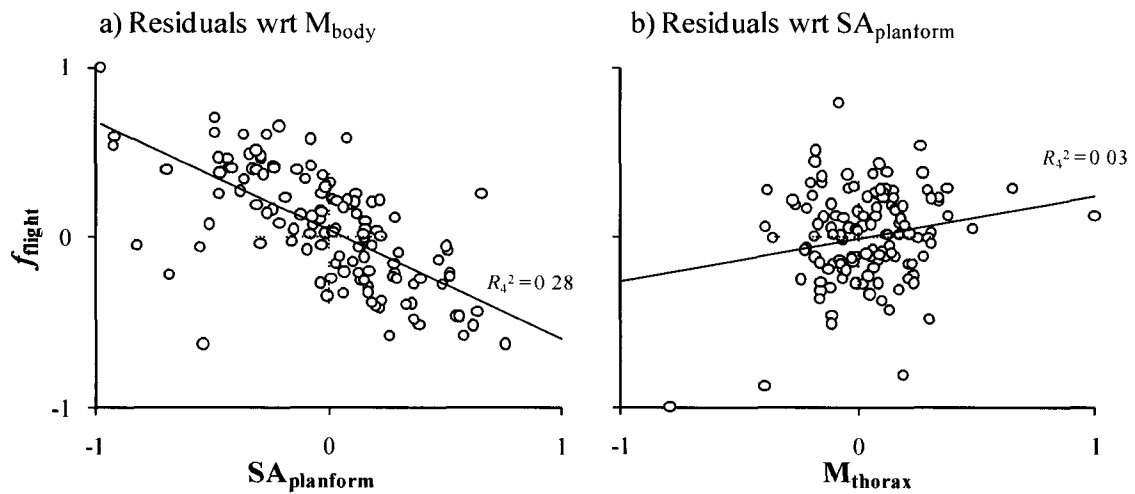


Figure 3.2 Influence of body size idiosyncrasies on wing beat frequency (f_{flight}) during flight. The residuals of f_{flight} on M_{body} are plotted against SA_{planform} (a) and the residuals of f_{flight} on SA_{planform} are plotted against M_{thorax} (b). Flight quality and colony are taken into account in both cases. Data are normalised to the range (-1,1).

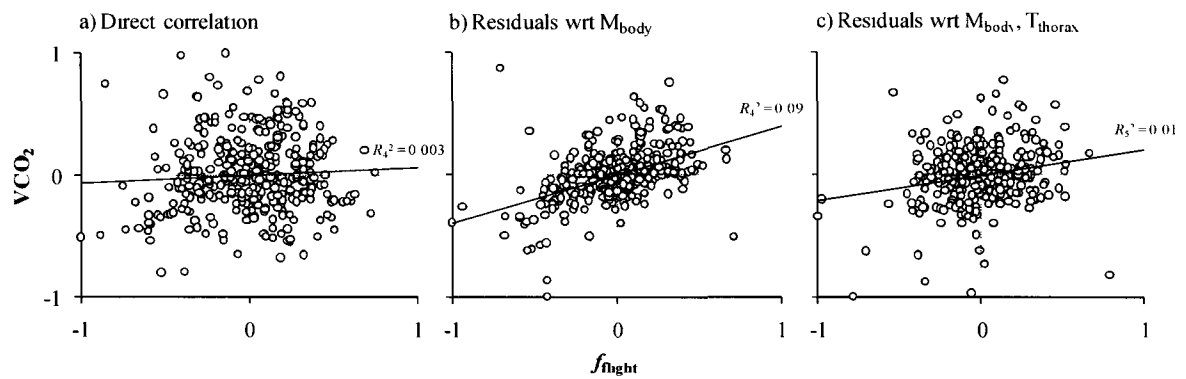


Figure 3.3 Comparison of absolute (a) and body mass (M_{body})-independent (b) correlations of f_{flight} and metabolic rate (VCO_2). The correlation is additionally shown after controlling for covariation with thorax temperature (T_{thorax} , panel c). Data are shown as residuals with respect to (wrt) flight quality and colony, as well as the factors indicated on the figure, and normalised to the range (-1,1).

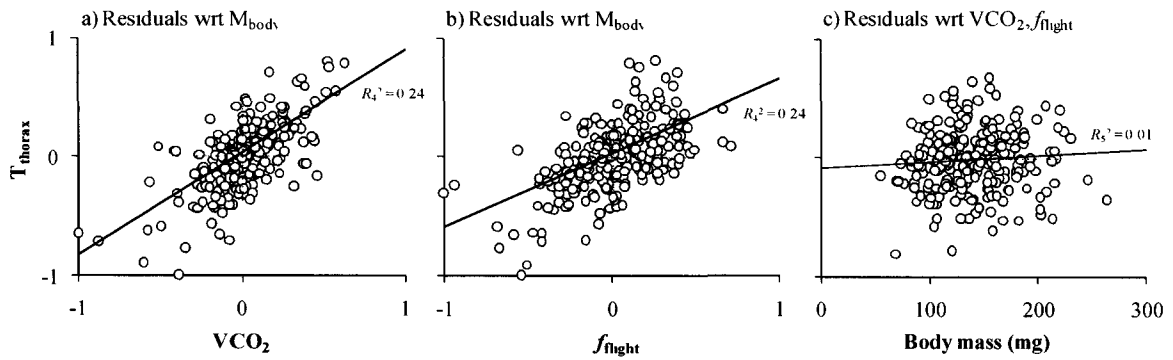


Figure 3.4 Covariation of thorax temperature (T_{thorax}) with metabolic rate (VCO_2 , panel a) and wing beat frequency (f_{flight} , panel b). The small amount of variation in T_{thorax} explained by body mass (Table 3.1), disappears after taking into account covariation with VCO_2 and f_{flight} (panel c) Data are shown as residuals with respect to (wrt) flight quality and colony, as well as the factors indicated on the figure, and normalised to the range (-1,1).

a) Direct correlations

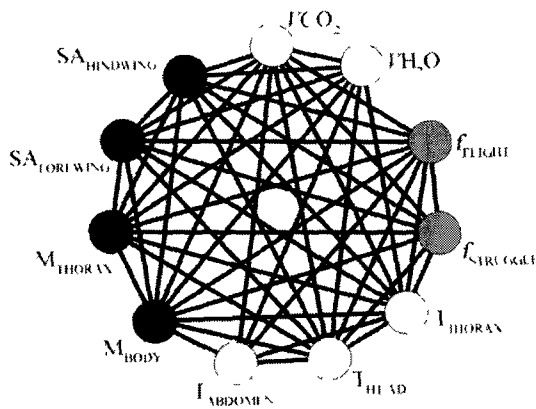
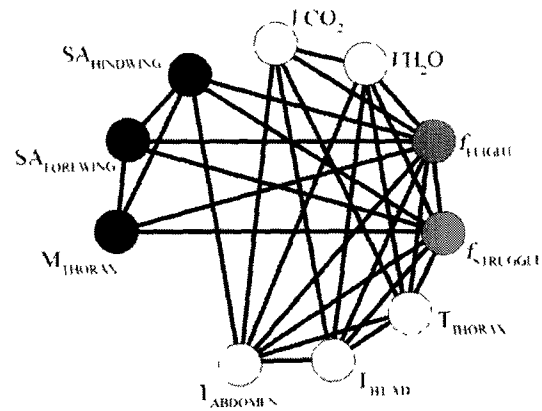
b) M_{body} -independent correlations

Figure 3.5 Graphical summary of the direct (a) and mass-independent (b) correlations among measures of individual body size and physiology. Each variable is a node of the graph, and edges represent statistically significant relationships. Nodes are shaded according to functional grouping: morphology (black), metabolism (white), wing beat frequency (dark grey), temperature (light grey). Controlling for mass reduces only a few sources of covariation.

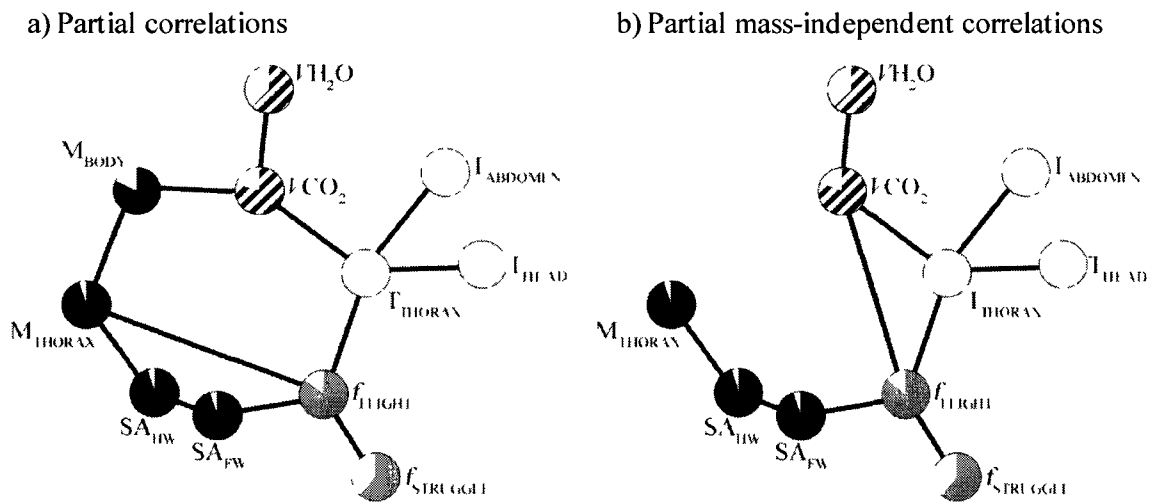


Figure 3.6 Undirected dependency graph obtained from the partial (a) and mass-independent partial (b) correlations among measures of individual body size and physiology. Each variable is a shaded node of the graph, and edges represent statistically significant relationships. The colour of the shade denotes functional groups: morphology (black), striped (metabolism), temperature (light grey), wing beat frequency (dark grey). The shaded areas for each variable represent the fraction of total variance (adjusted R^2) explained by the set of edges leading to that node, with the exception of body size measures, which were not predicted from flight parameters.

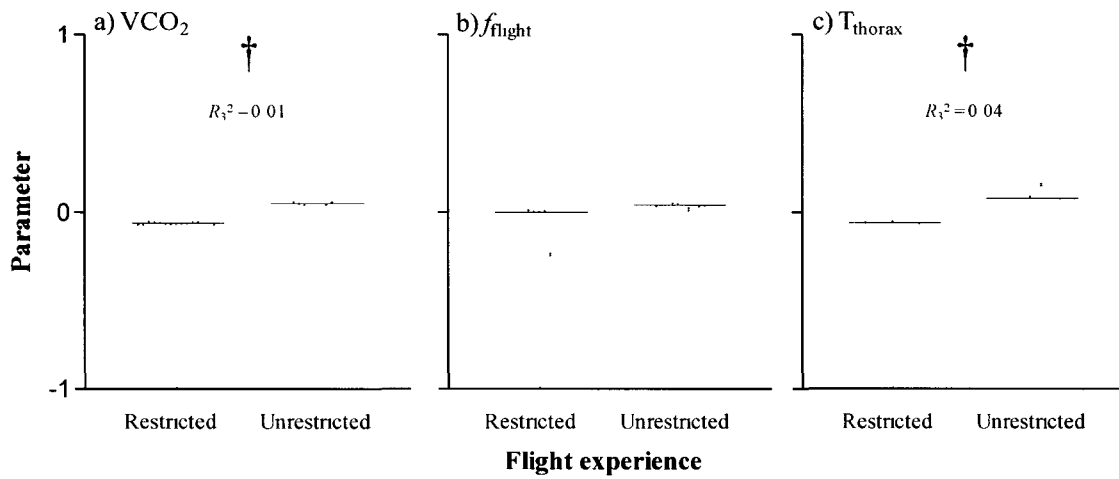


Figure 3.7 Effect of flight experience on metabolic rate (V_{CO_2}), wing beat frequency (f_{flight}), and thorax temperature (T_{thorax}). Each individual is represented by one circle. Significant differences ($p < 0.001$) in the mean are indicated by a dagger (†). Data are shown as residuals with respect to (wrt) flight quality, and normalised to the range (-1,1).

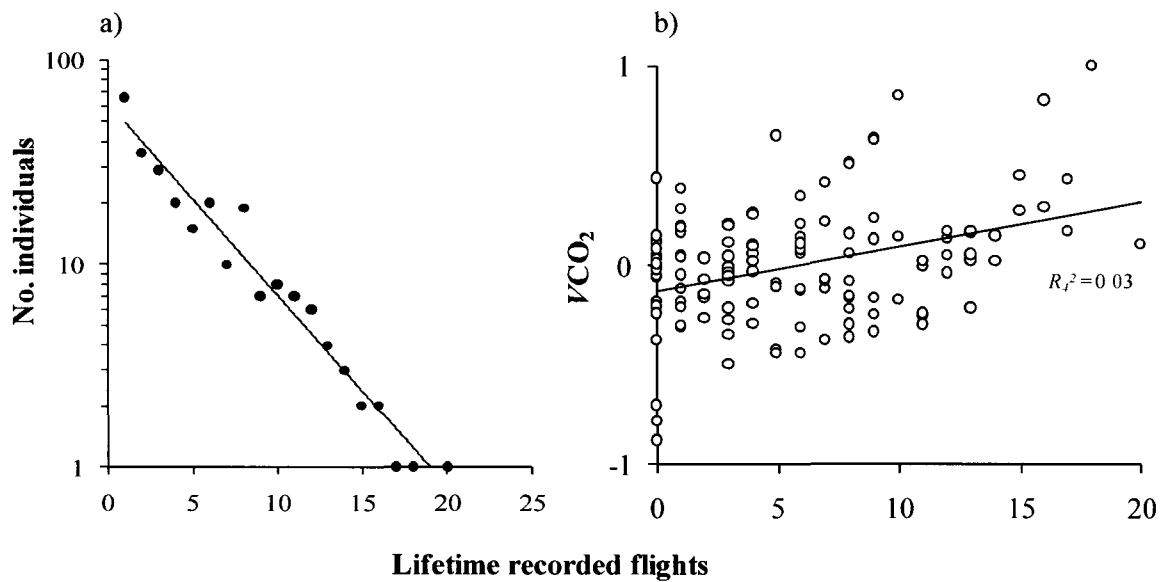


Figure 3.8 Distribution of lifetime flight experience (panel a) and its impact on metabolic rate (VCO₂, panel b). Each flight is a recorded guarding sortie in response to a disturbance of the colony. Metabolic rate is expressed as the partiresiduals after controlling for flight quality, colony, and body mass. Data are shown as residuals with respect to (wrt) flight quality, colony, and body mass, and normalised to the range (-1,1).

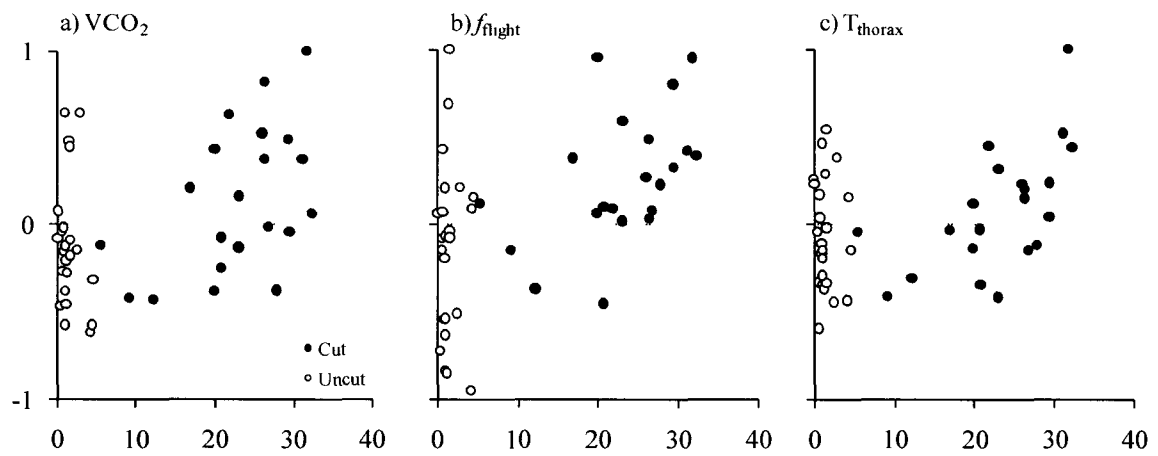


Figure 3.9 Effect of induced wing asymmetry on metabolic rate (V_{CO_2} , panel a), wing beat frequency (f_{flight} , panel b), and thorax temperature (T_{thorax} , panel c). Data are shown as residuals with respect to flight quality, body mass, and number of lifetime flights, and normalised to the range (-1,1).

CHAPTER 4:
**METABOLIC ORGANISATION IN WORKER BUMBLEBEES (*BOMBUS*
IMPATIENS) II: BIOCHEMICAL PLASTICITY AND METABOLIC
COVARIATION**

ABSTRACT

Correlated evolution of metabolic supply and demand occurs in all species, and has been hypothesised to be a driving force for diversity in physiology. However, novel phenotypes arising through natural and artificial selection often impact a limited number of physiological variables, while others show little or no change. This is surprising, since studies of physiological variation within populations suggest strong associations amongst enzyme activities and metabolism, so a change in one parameter would be expected to result in changes to others, simply by association. An integrative study of flight and enzyme capacity in orchid bees (Apidae: Euglossini) found that species exhibit correlated evolution of wing beat frequencies, metabolic rate, and just one enzyme among those measured, hexokinase. To support the conclusion that these changes represent evolutionary adaptations to increasing size and thus metabolic work, I examined whether similar patterns are observed intra-specifically. I examined inter-individual variability in enzyme activities of workers of *Bombus impatiens* (Apidae: Bombini), including reactions catalysed during energetic supply, glycolysis, and the tricarboxylic acid cycle. I then compared both covariation in enzyme activities, and between enzyme activities, body size, and metabolic rate. Among measures of body size, only thorax mass was predicted to explain variation in enzyme activity, and only in one enzyme, hexokinase, which decreased with increasing size. In turn, variation in other enzymes' activities could be partly explained by associations with hexokinase or other enzymes. Enzyme activity did not vary directly with metabolism, meaning that whole-animal physiology and cellular energetics are primarily linked by properties of the thorax, such as the mass of muscle. Experimental manipulation of flight history and flight costs did not lead to changes in activities of the measured enzymes, although there were differences in metabolic rates. These patterns are consistent with interspecific variation in the orchid bees,

suggesting that there may be a mechanism of plasticity that is common at least in the subfamily Apinae.

INTRODUCTION

Phenotypic plasticity allows individuals to acclimate to novel environments or conditions. In insects, muscle plasticity has largely been studied in conjunction with life history changes, generally with respect to the cost of maintaining metabolically expensive tissue (Marden 2000). However, such changes are highly regulated, which suggests changes in lifetime enzyme concentration are coevolved patterns rather than immediate responses to demand (Collatz *et al.* 1981; Chesky 1974). It is unknown how responsive adult insect muscles are to environmental differences in general, let alone whether plasticity is a common trait or has evolved specifically in different lineages. Acclimation experiments tend to focus on raising insects through their whole developmental period (e.g., Frazier *et al.* 2008), rather than in the adult stage alone. Due to their short life times, adults are probably considerably less plastic than previous life stages, and may show asymmetric responses to high and low acclimation temperatures (Terblanche *et al.* 2005) or possibly none at all (Lachenicht *et al.* 2010).

Plasticity also allows individuals to respond to variation in power requirements by remodelling muscles or elevating enzyme activities. Vertebrate muscles respond to training by elevating catabolic capacity (reviewed by Johnston & Moon 1980). In honeybees, muscle plasticity is apparently predictive of flight effort rather than dependent on experience (Schippers *et al.* 2010), and occurs with hormonal changes (Wolschin & Amdam 2007). Dealation in the already flightless bug *Pyrrhocoris apterus* triggers histolysis of the flight

muscle by accelerating hormonally-mediated behavioural changes, partly in response to the injury itself (Socha & Sula 2008). A potentially more direct approach to measuring plasticity is to measure response to flight experience, and in this case results are mixed. There is no change in the rate of actomyosin ATPase loss when houseflies, *Musca domestica*, are dewinged (Chesky 1974). In contrast, *Drosophila melanogaster* raised in confinement had lower citrate synthase activity and altered membrane composition (Magwera *et al.* 2006). Somewhat similarly, denervated *Locusta migratoria* flight muscle loses intermediate metabolic enzyme activities specific to flight, with compensation by elevated lactate dehydrogenase activity (Koster and Beenackers 1979). Forced exercise of maturing tsetse flies, *Glossina morsitans*, between blood meals, results in large changes in mitochondrial volume (Anderson and Finlayson 1976).

Intra-specific plasticity is accordingly at least twofold. First, individuals must acclimate to environmental influences, particularly temperature and humidity. Second, individuals must respond to work and load requirements. These latter requirements may arise from intra-specific variation and idiosyncrasies and exercise training (Chapter 3). Moreover, it may be that insect muscles are not equally plastic throughout their lifetimes since transcription and translation only occur early in adult life (Chapter 2). I previously examined the time course of enzymatic maturation in bumblebee (*Bombus impatiens*) workers, and found they begin flight prior to full biochemical maturation. This seems to be broadly true of insects (e.g., Gunn & Gatehouse 1988; Collatz *et al.* 1981). Possibly, there could be a window of plasticity after emergence, between the start of flight and the gain of full enzymatic competency. In this period, new adults might be able to adjust metabolic supply to match demand caused by idiosyncrasies in development. If this is the case, and

muscle plastically responds to work load, then restricting individuals from flight should lead to lower enzyme activities. Preventing bumblebees workers from flying led to differences in flight parameters (Chapter 3), which suggests concurrent changes in biochemistry.

There is also a question of how predictable are traits between species, from patterns within species. In *Drosophila*, species that have large pools of triacylglycerol also have large pools of glycogen, which mirrors the pattern between individuals in *Drosophila melanogaster* (Clark & Wang 1994). Intra-specific variation can thus lead to observed species differences. In orchid bees, mass-specific metabolic rates and wing beat frequencies decrease with body size, a decrease that is almost precisely matched by lower activities of hexokinase, the enzyme that commits glucose to glycolysis (Darveau *et al.* 2005b). Energy supply thus paces energy demand across species. Do similar patterns exist within species? In *Bombus impatiens* workers, body mass varies five-fold, although mass-specific metabolic rate and wing beat frequency do not scale with similar exponents (Chapter 3). This difference in scaling exponents suggests that although biomechanical principles may be similar across species, their impact may differ within and between species. It is less clear how similar biochemical patterns must be within species as between, since evolutionary time allows for more enzyme-specific selection or drift. The focus of this Chapter is therefore to elucidate whether biochemical patterns are similar to those in orchid bees and honeybees (Darveau *et al.* 2005b; Suarez *et al.* 1996). In particular, previous work has found that hexokinase activity decreases in parallel with metabolism, while other enzymes do not differ. One might expect, through a variety of mechanisms, that other enzymes would change by species, if only because their activities correlate with that of hexokinase within a

muscle. I therefore examine a number of enzymes to see how enzyme activities correlate within an individual.

I analysed enzyme activities of flown *B. impatiens* workers (Chapter 3) to compare enzyme activities to flight parameters. I examined correlations between enzyme activities and between enzyme activities and metabolic rate, to find if enzyme capacity covaries with any other trait better than expected by body size alone. In total, I surveyed eight enzymes, which allows me to evaluate whether enzymes are better (or only) correlated with adjacent biochemical reactions that share a substrate. I then compared enzyme activities between bees with different flight histories, to observe any effects of training on enzyme capacity. Finally, the results were compared overall to orchid bees, to assess the similarity of interspecific and intra-specific variation.

METHODS

Individuals

Individuals for enzyme assays were first selected based on the quality and availability of metabolic data (Chapter 3). Priority was given to individuals with high quality flights and surface temperature data. Insofar as possible, individuals were restricted to 19-24 days of age to maintain a mean of 21 days, with a final sample size of 133 individuals (68 unrestricted and 65 restricted). Additionally, 22 individuals with cut wings were processed. A subset of the enzymes tested in Chapter 2 was chosen for analysis, representative of major elements of the carbohydrate catabolic system: substrate mobilisation: trehalase (TRE); glycolysis: hexokinase (HK), phosphoglucose isomerase (PGI), aldolase (ALD), enolase (ENOL), pyruvate kinase (PK); NADH regeneration: α -

glycerol-3-phosphate dehydrogenase (α GPDH); tricarboxylic acid cycle (TCA): citrate synthase (CS). Assay conditions were described in Chapter 2.

Partial flux velocity

Metabolic rates (V_{CO_2}) were converted to a biochemically relevant estimate of substrate flux rates (v) in order to characterise the velocity through particular reactions. The calculation has been described previously (Suarez *et al.* 1996; Staples & Suarez 1997). Whole-animal V_{CO_2} was converted to VO_2 assuming a respiratory quotient equal to one, as in other bees (Suarez *et al.* 2005), and then reported relative to thorax mass (M_{thorax}), which is the primary location of flight metabolism. Since the thorax contains both flight muscle and cuticle, M_{thorax} was multiplied by 0.885 to approximate flight muscle mass (Buchwald & Dudley 2010), and then by 0.63 to find the mass of water (Staples & Suarez 1997). mL O_2 was converted to moles at 24°C (lab ambient temperature) and one atmosphere pressure. The partial flux velocity was expressed as v/V_{max} .

Data analysis

The statistical methods were described in Chapter 3. For correlations with enzyme activity data, day of assay and colony of origin were included as covariates. Where flight and enzyme data are considered simultaneously (such as during partial fluxes), flight quality was also included as a covariate. Individuals with cut wings were excluded from initial analyses, and when examined, only body mass was taken as a covariate, as all individuals were from one colony. All correlations were tested with linear models ($\alpha=0.05$), and reported F-statistics have degrees of freedom in subscript.

RESULTS

Variation in Enzymatic Activity

Three parameters of body size were included to discriminate between different measures, as described in Chapter 3, with the exception that I used the surface area of the wing planform (SA_{planform}) rather than discriminating between the hind- and forewings. Relationships between enzymes and body size were contrasted between those observed by direct correlation (Fig. 4.1a) or partial correlation (Fig. 4.1b). Partial correlations greatly reduced the number of significant relationships, but no variable was orphaned. Total body mass (M_{body}) and SA_{planform} were best correlated to thorax mass (M_{thorax}) alone, not each other. All enzymes were directly correlated with other enzymes, however only HK, PK, and CS were correlated with any of the three measures of body size, and only HK with all three (Fig. 4.1a). After accounting for covariation, only HK remained significantly related to body size (Fig. 4.1b). HK is also prominent for being the most connected enzyme after accounting for covariation (Fig. 4.1b). Of the four enzyme pairs catalysing sequential reactions (TRE-HK, HK-PGI, ALD- α GPDH, and ENOL-PK), three are correlated.

Although HK correlated with body size ($F_{1,148}=13.78$, $p<0.001$), this explained only ~6% of the variance in enzyme activity (variation in HK with respect to M_{thorax} is shown in Fig. 4.2b). I therefore examined the adjusted model R^2 of each trait as a function of its linked variables. About 50% of the variation in HK was explained by its correlations to M_{thorax} , TRE, PGI, ALD, ENOL, and CS. Conversely, only 6% of the variance in TRE was explained by HK and ALD activities. The fraction of explained variance for each trait is overlaid in Fig. 4.1b.

Because mass explains so little variance in enzyme capacity, it is important to examine how well coordinated enzyme activities are within individuals. One method is to compare residuals of enzyme activities with respect to body size (Fig. 4.3). Corroborating the graph of partial correlations, most enzymes increase with increasing HK (other pairs of correlations not shown). Moreover, individuals that appear as outliers when comparing mass to enzyme activity, can be identified to determine whether their activity is at least consistent between enzymes. For example, one 50 mg individual with particularly low HK activity for its mass (Fig. 4.2a and b), nonetheless shows low enzyme activity across the board (Fig. 4.3, datum at residual $HK=-1$).

Manipulation of Flight History

Individuals from colonies restricted from flight throughout their lifetimes were compared to those unrestricted but still stressed. I report the response of HK to treatments as representative of the enzymes in general (the observed patterns did not differ). There was no difference in HK between treatments ($F_{1,126}=0.93$, $p=0.34$; Fig. 4.4a). This might be because the lifetime number of flights varied extensively (Chapter 3, Fig. 3.8a), so a change would be most pronounced with more flights. Examining only the unrestricted individuals, there was no change in HK with number of flights (Fig. 4.4b), even if allowing for covariation in M_{thorax} and TRE activity ($F_{1,118}=0.49$, $p=0.48$). Finally, I examined whether clipping one wing and inducing asymmetry resulted in changes in enzymatic organisation. Only individuals from the treatment colony were used for comparison, and there was no change in HK ($F_{1,43}=0.21$, $p=0.65$; Fig. 4.4c).

Partial flux through hexokinase

Because there was no difference in enzyme activity during training, despite the change in metabolic rate (V_{CO_2}), I examined variation in partial flux velocity to see if unrestricted bees were more challenged. Since HK changed with M_{thorax} , and is thought to operate at a high fraction of maximum capacity in honeybees (Suarez *et al.* 1996), I examined flux through this step (HK_{flux}). The partial flux velocity was compared to M_{body} . Both HK and V_{CO_2} decrease with size, so this distinguished whether one or the other decreased more quickly. There was no correlation to M_{body} (Fig. 4.5a, $F_{1,108}=0.98$, $p=0.32$), indicating that changes in V_{CO_2} are paced by lower HK.

Between individuals, relative variation in flight parameters could influence flux. This is especially true of temperature in the muscle, which generally affects chemical kinetics. Compared absolutely, there was a strong positive correlation between HK_{flux} and individual body temperature (Fig. 4.5b, $F_{1,102}=35.52$, $p<0.001$). The restricted and unrestricted individuals fell along the same line (no difference in intercept: $F_{1,101}=1.00$, $p=0.32$), but the former were shifted to the lower end, and the latter to the upper end. Across all individuals, when an individual had higher T_{thorax} than predicted by M_{thorax} , then its HK_{flux} was also higher (Fig. 4.5c, $F_{1,101}=60.80$, $p<0.001$). The difference in intercepts by flight experience was not significant ($F_{1,100}=0.01$, $p=0.93$), indicating the shift in temperature was likely alone responsible for the increased HK_{flux} .

Integration of whole-animal and muscle physiology

These results suggested that enzyme V_{max} and flight parameters are not linked to a greater extent than predicted by body size. I examined partial correlations among all data

(Fig. 4.6). The paths do not suggest any further sources of shared variation across biological levels. VCO_2 and wing beat frequency (f_{flight}) only predicted HK by covariation with M_{thorax} , but M_{thorax} still predicted HK after accounting for VCO_2 and f_{flight} ($F_{1,111}=7.91$, $p=0.006$).

DISCUSSION

Phenotypic plasticity is the appearance of variant phenotypes in identical genomes, or even identically regulated genomes. Physiological plasticity arises from an individual's interactions with its environment or its lifetime experience. I found that the activities of three enzymes, HK, PK, and CS, changed with body size (Fig. 4.1a), but for PK and CS this was not more significant than covariation with other enzymes (Fig. 4.1b). As in orchid bees (Darveau *et al.* 2005b), only HK correlated to body mass, and HK activity decreased with larger size (in contrast, Newsholme *et al.* 1972 did not find any change in HK with body size in *Bombus sp.*). HK was correlated with most other enzymes' activities, or was linked to others through a common partner (Fig. 4.1b). Because HK appears to be so central within this network, it reinforces the view that HK is particularly crucial for coevolution of metabolic supply and demand in orchid bees (Darveau *et al.* 2005b). This also suggests that the evolution of HK within the orchid bees is not an extension of intra-specific plasticity. Although the pattern is the same in both cases, if plasticity alone accounted for the change in activity, other enzymes should have changed in parallel. The origin of correlations between enzyme activities and the scaling of enzymes with body size is poorly understood. Evidence from fish suggests that correlation and scaling cannot be explained, or at least completely explained, by transcriptional variation (Burness *et al.* 1999; Davies & Moyes 2007; Yang & Somero 1996). Moreover, the correlation between enzymes depends on the tissue that is

studied (Garland 1984), though in that respect, insect flight muscle provides some advantages for study.

HK correlated to body size (Fig. 4.2b), and in turn, other enzymes were strongly correlated with HK (Fig. 4.3). Interestingly, when predicting HK activity, M_{thorax} did not explain as much variation as enzymes, especially TRE and PGI. In general, activities of enzymes within glycolysis were the best predicted, and around ~30% of their activity could be explained on the basis of other enzymes in the pathway. Enzymes which are not members of glycolysis, TRE (substrate mobilisation), CS (TCA), and α GPDH (NADH regeneration), were the most poorly predicted (6% of their variation). This is partly the result of which enzymes were selected, but it suggests that different pathways may be regulated as separate units. Each unit may be organised to a certain extent, and then within that unit, certain steps may be additionally refined (plastically or evolutionarily). Additional research may identify how much of the variation is due to functional requirements or another evolutionary process, such as drift. For instance, interspecific comparisons of correlated metabolic enzyme activities in the genus *Drosophila* (Clark & Wang 1994) and in the fish genus *Fundulus* (Pierce & Crawford 1997), indicate that some of the variation is nonadaptive, at least in relation to metabolic rate and temperature adaptation.

Evolution of glycolytic enzyme activities may also occur because of their participation in non-metabolic functions (e.g., glyceraldehyde-3-phosphate dehydrogenase, Sirover 1999), but I assume here that flight muscle enzyme capacity evolved primarily to fuel flight metabolism (Suarez 2000). This might suggest that metabolic organisation is designed to prevent over- or under-matching of adjacent biochemical reactions, which could lead to excess or bottle-necking of pathway activity. In this case, enzyme capacities of

sequential reactions should be correlated with each other, which has occurred in the evolution of the genus *Drosophila* (Clark & Wang 1994). My selection of enzymes precluded explicitly testing for a bias toward correlations of adjacent enzyme reactions, although three of four pairs of enzymes catalysing sequential reactions were correlated (Fig. 4.1b). However, the converse hypothesis, that non-adjacent enzymes are not correlated to each other, was not observed (Fig. 4.1b).

Correlated enzyme activities may also be important because enzyme complement alone is insufficient to predict flight ability (Wojtas *et al.* 1997). Insect muscles have evolved more rather than better enzymes (Suarez 1998) and are physically more efficiently spatially designed than other organisms (Iwamoto *et al.* 2006). Insect mitochondria are at the limit of their packing density in the cell (~40% volume; Suarez 1996), so cells may also have evolved more efficient enzyme localisation, which affects how efficiently substrate is transferred from one step to another. Glycolytic enzymes localise to the Z- and M-bands of the muscle fibre, and use information from at least one other enzyme, α GPDH, to distribute properly (Sullivan *et al.* 2003). Recent evidence in plants (*Arabidopsis thaliana*) suggests that the localisation of glycolytic enzymes to the surface of the mitochondria can be actively regulated by oxygen consumption (Graham *et al.* 2007). It should be examined *in vitro* whether insect fibres and mitochondria are capable of increasing enzyme concentration without structural consequences.

In response to exercise, the bees elevated their metabolic rates (Chapter 3), even though no change in enzyme capacity at either HK or CS was observed. I had hypothesised that changes in HK activity were the most likely, because of its correlated evolution with metabolic demand in orchid bee species (Darveau *et al.* 2005b) and because I found HK was

correlated to M_{thorax} (Fig. 4.1b). CS was another candidate to change, as honeybees may increase CS in anticipation of foraging (Schippers *et al.* 2010), and because CS is a marker of mitochondrial density. Since there were no changes in enzyme activity, an alternative possibility is that bees behaviourally raised T_{thorax} , allowing for higher velocity of substrate through glycolysis behaviourally by elevating T_{thorax} (Fig. 4.5b). This might be the case if the inhibition of HK by ATP was reduced by higher temperature, or if other biochemical effects acted to increase the activity of HK (Scaraffia & Gerez de Burgos 2000). This plasticity might be a behavioural alternative to costly translation of more enzyme. However, there are statistical problems with comparing ratios like HK_{flux} , which depends on M_{thorax} through VCO_2 and HK activity, to traits like T_{thorax} that also depend on M_{thorax} . HK_{flux} did not change directly with M_{body} , so I cautiously suggest the relationship of HK_{flux} and T_{thorax} is not a simple autocorrelation.

The degree to which metabolic supply matches demand has been extensively studied in vertebrates, which readily respond to acclimation. In insects, whose muscles might be less plastic (see Introduction), flight phenotypes are influenced by genotype, such as the well-documented variation in malate dehydrogenase alleles in honeybees (Harrison *et al.* 1996a) and PGI alleles in butterflies (Niitepõld 2010). Within *Drosophila melanogaster*, 9-35% of variation in metabolic rate can be explained by genotypic mapping (Montooth *et al.* 2003). The bees in this study are expected to have comparatively low genetic variation, and on average, 75% related siblings, provided a monogamous mother (Payne *et al.* 2003). If a genetic component was present here in determining enzyme activity, it could not be excluded from the effects of mass alone. On the other hand, my own observations suggest bumblebee

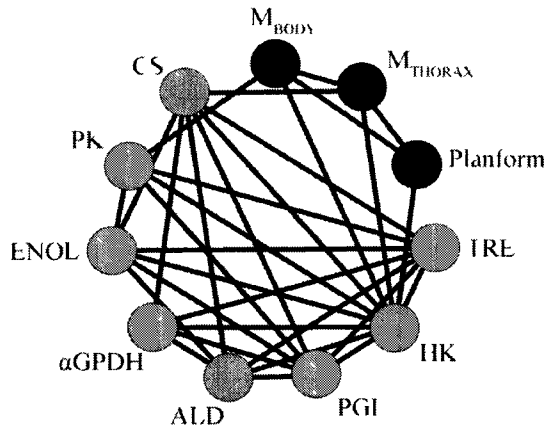
queens raised in greenhouses are not necessarily monogamous, so intercolony variation may not be a good measure.

Variation in HK was also not attributable to plasticity in response to training and elevated metabolism. After accounting for M_{body} and evolutionary relatedness among orchid bee species, Darveau *et al.* (2005b) found that HK and metabolic rate were not correlated. In *D. melanogaster*, metabolic rate was weakly, if at all, associated with enzyme activity (Laurie-Ahlberg *et al.* 1985). Together, this suggests that enzyme activities are more correlated with each other than to functional requirements (see also Clark & Wang 1994; Pierce & Crawford 1997). In general, enzyme activities may be established according to a general body plan, and morphology in turn is related to metabolic rate (Fig. 4.6). Interestingly, HK and f_{flight} both scaled with body mass to the same exponent ($\sim M_{\text{body}}^{-0.10}$), while mass-specific $V\text{CO}_2$ decreased more quickly ($M_{\text{body}}^{-0.25}$). In contrast, all three variables scaled equivalently in orchid bees ($M_{\text{body}}^{-0.3}$; Darveau *et al.* 2005b). This suggests that the latter evolutionary patterns may have resulted from modification of the underlying intra-specific plan. By extension, within each orchid bee species, the relationships of HK, f_{flight} , and $V\text{CO}_2$ to M_{body} should differ in intercept, but not in slope.

These results reveal that intraindividual biochemical capacity is highly organised, potentially with respect to a particular enzyme, HK (in *Drosophila*, the organising enzyme appears to be αGPDH , Sullivan *et al.* 2003). The extent of explainable variation in enzyme activity was on average 30%, but often as little as 6%, depending on whether the enzyme was involved in glycolysis or another metabolic unit. Variation in enzyme activity does not appear to have consequences for metabolism in the steady, agitated flight studied here, since $V\text{CO}_2$ did not correlate with enzyme activity. The flux velocity through HK was only 50%

of enzyme capacity, so individuals have considerable safety margins. A better experiment for measuring the matching of supply and demand might be in more strenuous lift conditions, such as load-bearing or heliox (Buchwald & Dudley 2010; Roberts *et al.* 2004). There were some indications that by regulating body temperature, bees might be able to regulate flux rates. Such behavioural accommodation, which is cheaper than protein translation, might explain why I did not find training differences in enzyme capacity. However, it is difficult to conclude that the bees were extensively challenged by the short flights in this training paradigm. Future experiments should increase the intensity of exercise (more similarly to Anderson & Finlayson 1976). This may reveal the extent of underlying biochemical plasticity, if it exists.

a) Direct correlations



b) Partial correlations

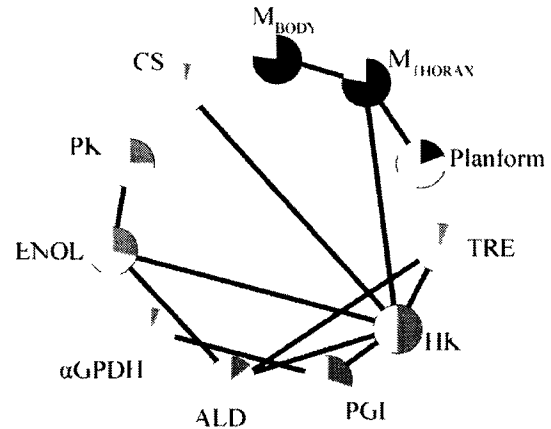


Figure 4.1 Direct and partial correlations between body size and metabolic enzyme activity. Nodes represent variables and edges represent significant pairwise (direct or partial) correlations. For each variable, the fraction of explained variance (shaded slice) was examined with respect to the contribution of all variables connected by its set of edges (e.g., CS predicted from HK). The exception was the thorax mass (M_{thorax}), whose variation was not predicted from enzyme activity.

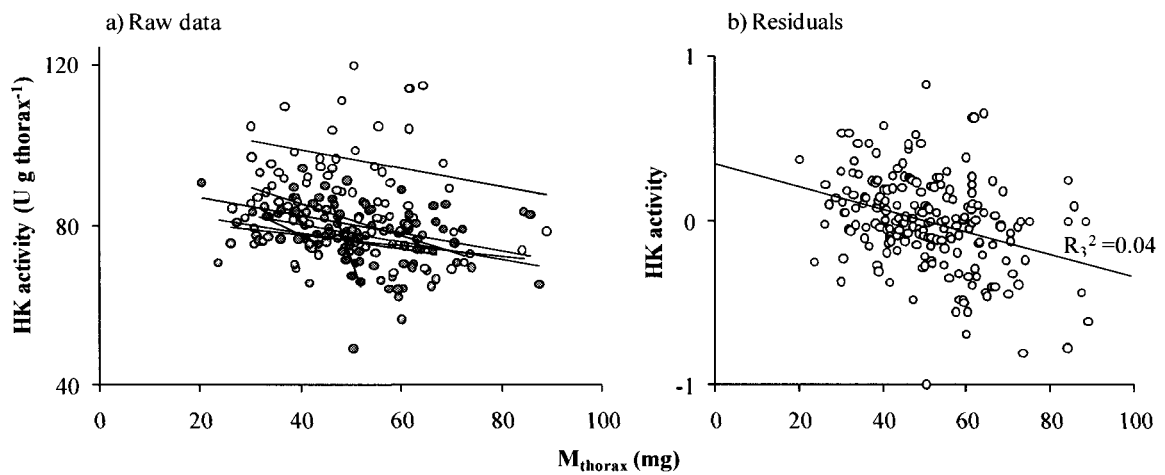


Figure 4.2 Correlation of hexokinase (HK) activity to thorax mass (M_{thorax}). Raw HK activity (panel a) is presented for comparison of day-of-assay effects, and overall, HK decreases with M_{thorax} (panel b). Data are shown as residuals with respect to day of assay, and normalised to the range (-1,1). R^2 is partial with respect to day of assay and colony.

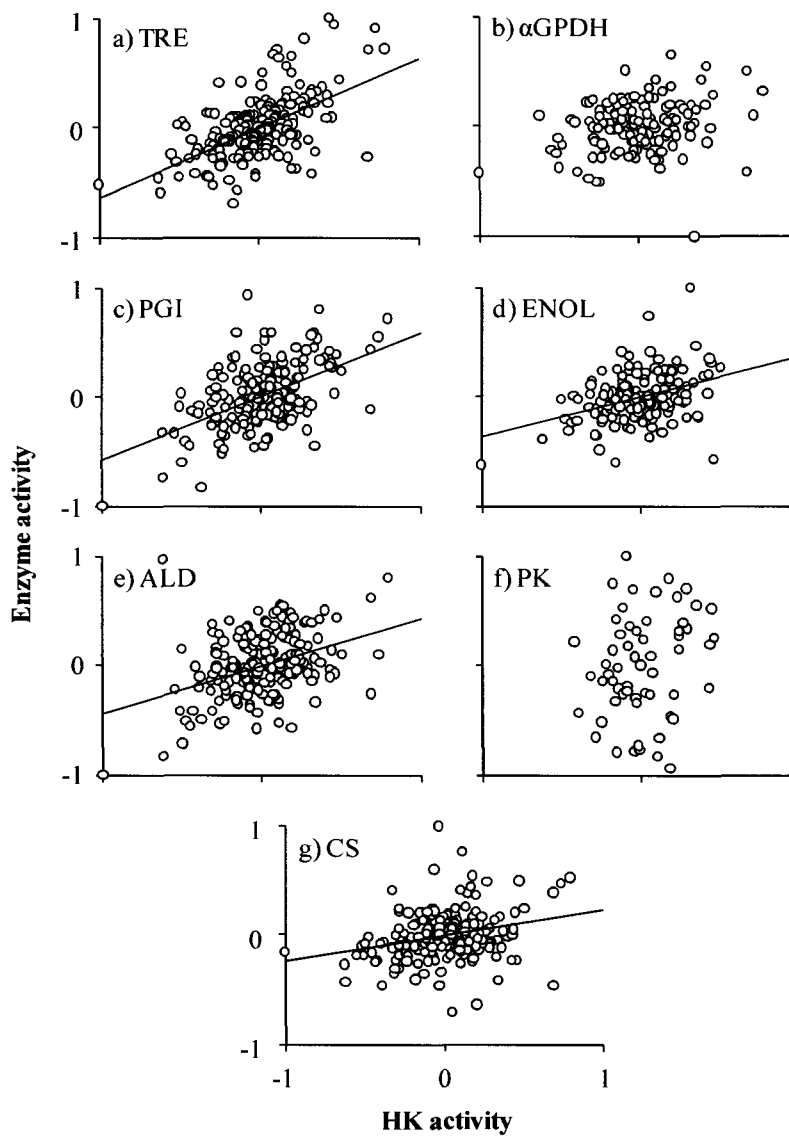


Figure 4.3 Mass-independent correlations of hexokinase (HK) to other enzymes' activities. Activities are presented as residuals with respect to day of assay and thorax mass, except ENOL, which is additionally with respect to colony, and then normalised to the range (-1,1). TRE: trehalase, α GPDH: α -glycerol-3-phosphate dehydrogenase, PGI: phosphoglucose isomerase, ENOL: enolase, ALD: aldolase, PK: pyruvate kinase, CS: citrate synthase.

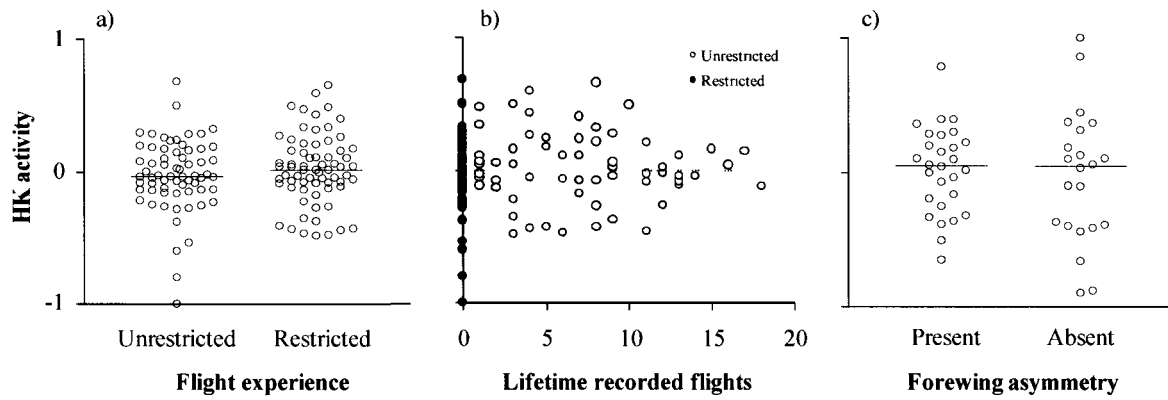


Figure 4.4 Hexokinase (HK) activity does not change with flight history. In panel a, HK activity was compared in experienced (unrestricted) fliers and inexperienced (restricted), and in panel b, HK was compared to the total number of lifetime recorded flights. Bees were also challenged by wing clipping at a young age (panel c). HK activity is reported as the residuals with respect to day of assay, and normalised to the range (-1,1).

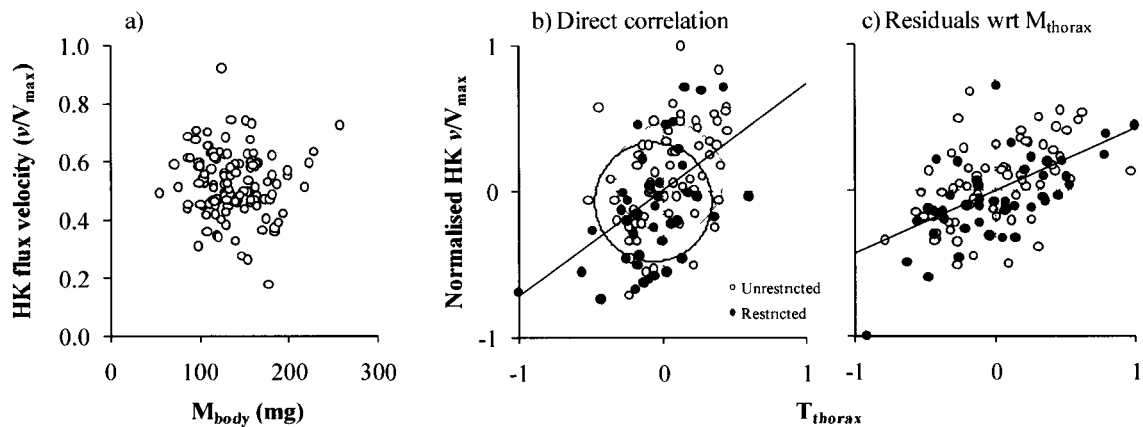


Figure 4.5 Flux velocity (v/V_{\max}) through hexokinase (HK) as a function of body mass (M_{body}) and thorax temperature (T_{thorax}). HK partial flux velocity did not change with M_{body} (panel a). However, higher T_{thorax} resulted in a greater flux through HK (panel b), and the positive shift of experienced fliers relative to inexperienced suggests the former may be taking advantage of this relationship. The centres of mass of the data, and the average radius of each point from the centre of mass, are shown as grey and black circle, for the unrestricted and restricted individuals, respectively. After accounting for covariation with thorax mass (M_{thorax}), individuals with relatively higher T_{thorax} also have relatively higher velocities (panel c). Regression lines are performed over all individuals, as there is no significant difference in intercepts in panel b or c. Data are shown as residuals with respect to (wrt) flight quality and day of assay, as well as the factors indicated on the figure, and normalised to the range (-1,1).

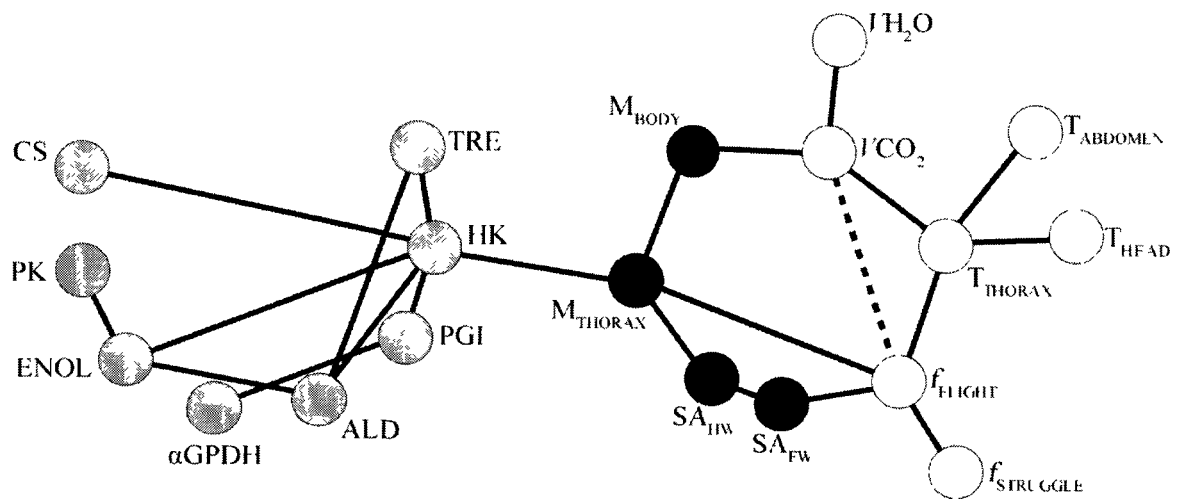


Figure 4.6 Correlations between body size (black), flight physiology (white), and enzyme activities (grey). Each variable is represented as a node, and edges represent significant partial correlations between variables. The dashed line indicates a significant mass-independent relationship.

CHAPTER 5:
ECOLOGICAL AND EVOLUTIONARY CONSEQUENCES
OF PLASTICITY AND VARIATION

Dimitri A. Skandalis

Biology is a continuum, but we biologists, because of our limitations, divide ourselves into categories, and then we pretend that those categories exist in the living systems that we study. From the functional point of view, of course, an animal is indivisible, and physiology is not in any sense an isolatable component of an organism. If physiology is defined as the study of vital functions, it becomes inseparable from morphology and behavior.

George A Bartholomew (1958)

C. Ladd Prosser outlined five goals of comparative physiology, and the third of these was *to provide the physiological basis of ecology...* (Garland & Carter 1994). Bartholomew, too, thought that the animal could not be fully understood apart from its environment and behaviour. In this thesis, I have discussed how inter-individual idiosyncrasies may lead to individual phenotypes: in a laboratory. However, these data shed light on an existing evolutionary question, the metabolic evolution of orchid bees. Darveau *et al.* (2005a, 2005b) observed a startling correlated evolution of body size, metabolism, wing beat frequency, and hexokinase activity within these bees. However, in order to conclude that the evolution is, in fact, correlated, one must first exclude the null hypothesis that the pattern is not the result of intra-specific plasticity. Put another way, one must be certain that there are evolutionary forces at work, and that scaling any one species to the size of another would not lead to the same observations. For this to be true, one of two conditions would need to be true. If the muscle phenotype is responsive to body size alone, then transplanting muscle from one bee to another would result in a new phenotype, based on the new body size. This is plasticity in the muscle growth, and is difficult to test. Another possibility is that all muscle generates a minimum of enzyme activity, and is then capable of developing the remainder based on

feedback from flight experience. Some oxygen consumption during flights of young individuals thus helps to set the adult phenotype. Because of this possibility, I examined the maturation profile in bees (Chapter 2), and found that they begin flight prior to full maturation. Consequently, there does indeed appear to be a period in which such feedback might occur.

In part, Darveau *et al.* tackled this by examining phylogenetic relatedness of bees' physiology. For some correlations, such as body size and metabolism or wing wing beat frequency, differences cannot be explained by evolution alone. This follows from Chapter 3, in which I suggest that the biomechanical rules governing physiology in this flight regime are quite general, and even explain variation between individuals. On the other hand, metabolism and wing beat frequency were not well correlated after taking phylogenetic relatedness into account. Since there is a relationship between these two variables intra-specifically, this suggests that evolutionary fine-tuning may be at work. Both inter-specifically in the orchid bees, and intra-specifically in the bumblebees (Chapter 4), there was no direct correlation between enzyme activity and metabolic rate. It appears that the two are most fundamentally linked by their correlation with body size (Fig. 4.1). This is reinforced by the observation that flight training has little effect on the muscle. Overall, it is therefore unlikely that intra-specific plasticity explains the variation between species, even though the patterns are similar. It is more likely that the intra-specific patterns are a general plan, which is modified over evolutionary time. However, it is obvious that the most thorough understanding of plasticity in orchid bees can only be obtained by studying orchid bees themselves.

In this Chapter, I will discuss both the limits to interpretation of the data in the thesis, and also how future research might provide them an ecological and evolutionary context. First, I will comment on some unresolved issues in muscle plasticity and design in bees, and then comment on likely path directions (causation) in the undirected dependency graphs that have been presented. Understanding these paths, and hence understanding which variables drive changes in other variables, is critical. With these paths, it is then also possible to suggest how maturation and development could be exploited by the insect to result in phenotypes appropriate to the environment. Finally, I suggest how inter-individual variation may have colony-level effects in bumblebees.

Metabolic organisation and influence of training

The lack of a training effect on enzyme activity might have been predicted because insects seem to be packing in enzyme near the limits of cellular spatial capacity (Suarez 1998; Wojtas *et al.* 1997). On the other hand, Schippers *et al.* (2006) found that aldolase and troponin T 10a protein concentrations, and citrate synthase (CS) activity, did increase in honeybees in anticipation of increased foraging effort. In tsetse flies and fruit flies too, flight experience has a measurable impact on mitochondrial capacity (Anderson & Finlayson 1976; Magwere *et al.* 2006). I think the most likely explanation is that the training paradigm was not challenging enough. The brief daily guarding flights amount to a very small fraction of the time a foraging adult might spend in flight. I suggested in Chapter 2 that insects might be most plastic in the period immediately after emergence, but my experimental design limited the testability of this hypothesis, because individuals that flew often necessarily started flying young (Fig. 5.1). In a statistical model comparing the effects of age at first

flight and lifetime flights on VCO_2 , age is not significant (Age at first flight: $F_{1,104}=1.59$, $p=0.21$; Number of flights: $F_{1,104}=4.94$, $p=0.03$). This suggests younger bees are not more plastic than older bees. But the collinearity between these two measures prevents strong confidence in that result, and so future experiments should compare young and old bees explicitly.

Honeybees might not make a good basis for comparison. In honeybees, hormones instigate both the behavioural transition from nest-work to foraging, and the accompanying changes in muscle phenotype (Wolschin & Amdam 2007). For insect species that exhibit dramatic plasticity in enzyme content with life history, hormonal signalling may be required. Bumblebees do not have a behavioural transition, though since bees become excited by high rewards (Mapalad *et al.* 2008; Moffatt 2001) foraging flights (as opposed to guarding flights) might result in biochemical changes. Besides this, adult honeybees' muscle plasticity may have evolved as a consequence of their colony structure. With tens of thousands of workers, colonies should seek to minimise the energetic investment per worker, which also means minimising each individual's excess enzyme capacity. Allowing individuals' flight muscles to change plastically with behaviours of different metabolic intensity ensures that the cost of transcription and translation is only borne when it is required. The corollary to this is that unladen honeybees operate at 75% of hexokinase (HK) capacity (up to 98% with load), while female bumblebees and male orchid bees (Darveau *et al.* 2005b), operate at 50% and 20% of HK flux capacity, respectively. As far as the close matching of enzyme content and metabolic rate (MR) goes, honeybees may not be generally representative, and might have been a serendipitous model for initial studies of low safety margins in enzyme capacity (Suarez *et al.* 1996; Staples & Suarez 1997).

If the muscle phenotype is indeed not plastic, and enzyme activities cannot be changed, it is possible that behavioural accommodation is possible. In Chapter 4, I presented tentative evidence that one benefit of elevated T_{thorax} might be increased enzyme flux. This suggests a loop: higher metabolic rates produce more heat, which in turn increases the operating temperature of the enzymes and allows for higher metabolic rates. By elevating temperature, the bee more efficiently uses the enzyme that it already possesses. The uncoupling of $V\text{CO}_2$ from wing beat frequency (f_{flight}) in the wing cut experiment may indicate that the increased energetic cost was not associated with the negligible increase in f_{flight} , but rather an increased cost of corrective steering and motor control. Reductions in maneuverability of flying insects with wing damage has been found (Combes *et al.* 2010; Jantzen & Eisner 2008).

From correlations to directed paths

Flight is a demanding performance not only because of the metabolic demands, but also due to the need to rapidly acquire, assimilate, and act on information about the environment (Candy *et al.* 1997). As a consequence, the variables in this study are a very small part of the flight experience of the bee. In this thesis I attempted to discover how parameters integrate only in one mode of flight at a constant temperature. As a result, the network of correlations that I have proposed (Fig. 5.2) may be limited in its wider application. There will be exceptions to nearly every proposed path, even without attempting to imply causation. For instance, as flight duration increases, M_{body} will decrease due to fuel consumption, so f_{flight} and $V\text{CO}_2$ will fall too (Hanauer-Thieser & Nachtigall 1995). Since only M_{body} changes among the morphological parameters, this links it to both

V_{CO_2} and f_{flight} . Other exceptions will probably only alter the correlations among the parameters, not the links themselves. For instance, genetic variation at enzyme loci alters the slope of MR on ambient temperature (e.g., Harrison *et al.* 1996a; Niitepõld 2010). In fact, these exceptions follow from the concept of the graph, which implies that flight control is chaotic, and therefore highly dependent on initial conditions. But because of this, I will only hypothesise directions to the paths in Fig. 5.2 assuming the same conditions in which the bees were tested.

There are three hierarchies in this discussion, colour-coded in Fig. 5.2. The static dimensions of body size (black nodes) are at the top of the hierarchy, and cannot be altered except by experimental manipulation (striped nodes). Flight parameters and enzyme activities (white and grey nodes, respectively) are affected by size and by each other.

The centrality of body size implies it is the intermediate between cell and whole-animal metabolism. Enzyme activity (V_{max}) is probably mostly genetically established by a characteristic body plan. This is corroborated by the fast maturation of V_{max} prior to flight (Chapter 2) and by the ability to discriminate with 97% accuracy between *Drosophila* species based on the enzyme complements of single individuals (Clark & Keith 1988). In addition to genetics, I hypothesised that plasticity might play a role in determining adult enzyme complement. I suggested that the metabolic information gained by an individual flying prior to maturing full enzyme capacity might be used to adjust capacity to match morphological idiosyncrasies. Individual variation in flight requirements was indeed observed, such as the contribution of relative wing area ($SA_{planform}$) to f_{flight} . However, I did not observe correlations between V_{CO_2} and enzyme capacity, either in the population as a whole, or after alterations with lifetime flights or wing asymmetry. While Montooth *et al.*

(2003) were able to explain about 30% of the variation in flight MR of *Drosophila melanogaster* by correlating it with genetic variation, Laurie-Ahlberg *et al.* (1985) found little or no association between V_{\max} and power output. Between orchid bee species, Darveau *et al.* (2005b) similarly did not find that V_{\max} and MR were more correlated than expected by body size. In addition, comparisons of enzyme activity to the initiation of flight during maturation show that individuals are able to fly with less than mature V_{\max} (Chapter 2). Further work with paired MR- V_{\max} measurements in maturing adults is needed to explain this. Overall, these results question to what degree V_{CO_2} is confined by enzyme concentration when the bee is not bearing loads.

The directionality of the paths between V_{CO_2} , T_{thorax} , and f_{flight} is very difficult to predict (Chapter 3) and so I think bidirectionality must be assumed for all the relationships. Further tests in which one component can be experimentally manipulated to examine correlated effects in the others, will help to elucidate directionality. For instance, the decrease in f_{flight} that has been observed with removal of the hindwing (Buchwald & Dudley 2010) would be expected to lead to reduced V_{CO_2} and T_{thorax} . Experimentally cooling or heating the thorax in flight may also be informative.

In terms of body temperatures, I think the path between T_{abdomen} and T_{thorax} is certainly bidirectional, since the sign of the relationship depends on whether the abdomen is passively being heated, or is actively cooling the thorax by heat shunting (Heinrich 1976). Whether this is also true of the head is more complex. It is possible that bees might be concerned with regulating head temperature, and so modulate T_{thorax} to prevent overheating (or overcooling). If T_{head} was regulated in these conditions, it would require thermoregulation through control of V_{CO_2} or f_{flight} , or evaporative heat loss. There is

already controversy over whether bees modulate metabolic rate in order to thermoregulate the thorax (Harrison *et al.* 1996b; Woods *et al.* 2005). At high ambient temperatures, V_{H_2O} in honeybees increases as evaporative water loss is recruited (Roberts & Harrison 1999). I found no sign of a correlation between V_{H_2O} and T_{thorax} , nor any sign of nonlinearities in their relationship at higher operating temperatures. The head's position near the thorax, and its lower surface area for heat dissipation compared to the abdomen, suggest that it is passively heated by the thorax. This suggests one reason why high thorax temperatures may be costly.

On the biochemical end of the spectrum, I do not see any way to predict the directions of the paths between enzyme activities, since we know very little about how cells regulate correlated enzyme activity. Subsequent enzymatic reactions are sometimes correlated with each other intra- and interspecifically (Clark & Wang 1994), but after accounting for covariation, I found many correlations among non-adjacent reactions. As far as mechanisms go, we only know that differential transcription does not explain correlated and uncorrelated changes in enzyme capacity (Burness *et al.* 1999; Davies & Moyes 2007), but the correlations do depend on the tissue that is studied (Garland 1984). Because of the bias towards linking HK and other enzymes, I am tempted to draw arrows out from HK. This might be true if it is at the core of a spatial organising principle, though this would then differ from *Drosophila*, which physically organises around an α GPDH anchor (Wojtas *et al.* 1997). It would be highly informative to examine whether correlations among glycolytic enzymes of *Drosophila* reflect this physical localisation, since genetic manipulation in *Bombus* would be more difficult.

Development and maturation in different environments

The paths in Fig. 5.2 reflect strategies that insects have evolved to deal with the effect of temperature on performance. At colder temperatures, insect f_{flight} and flight performance are reduced (Lehmann 1999; Frazier *et al.* 2008). In compensation, *Drosophila* pupae raised at lower temperatures develop larger wings and lower f_{flight} and power output (Frazier *et al.* 2008; Trotta *et al.* 2010). Enzyme activities follow suit: at lower temperatures *D. melanogaster* has reduced V_{max} (respiratory enzymes, Hunter & Cediell 1970). Interestingly, the number of individuals with wing abnormalities increases exponentially with raising temperature (Trotta *et al.* 2010), which will increase the variability of flight within the population. Variation in body size of *D. melanogaster* is not limited to temperature. Flies raised in different types of stressful environments develop different relative body size proportions, even while their mass is similar (Shingleton *et al.* 2009). In contrast to larger wings at lower temperatures, flies raised on poorer diets develop relatively larger thoraces and slightly smaller wings. This suggests a possible method to directly test the relative contributions of thorax mass and wing surface area to flight performance (Fig. 5.2; discussed in Chapter 3).

While adult flight performance is known to be altered by the environment in which larvae and pupae are raised, the amount of plasticity in the adults themselves is poorly understood. Although I suggested that the maturational period might afford individuals an opportunity to adjust cellular metabolism in response to idiosyncrasies in demand, I think it is more likely that plasticity in this period would be related to temperature. In *Bombus*, development time as a pupa may be two to three weeks long. The bee's environment might change radically between the start and finish of metamorphosis, and so an individual with at

least some amount of plasticity available when it emerges would be fitter in its adult environment. A recent examination of muscle enzyme plasticity in response to temperature from this lab found no signs of acclimation in mature adults (Licea, BSc Thesis 2010). Future work should compare whether newly emerged adults show more thermal plasticity than older adults. Of course, *Bombus* pupae are raised in thermoregulated colonies, so future work should also test if this differs between social and solitary bees, and other insects.

Functional consequences of individuality

Inter-individual variability may have impacts on bumblebee colony ecology, through variation in foraging efficiencies imparted by morphological and thermoregulatory idiosyncrasises. In bumblebees, larger individuals are more likely to forage, but it is unclear why individuals of the same size do or do not (Goulson *et al.* 2002; Jandt *et al.* 2009). Size-based hypotheses tend to rely on optimal energetic efficiencies, where the optimal size is that which maximises foraging returns to the energy intake of the individual (Pyke 1978): a worker that consumes more energy than she returns should switch to another task. Larger individuals carry more forage and may make faster round trips (Goulson *et al.* 2002). While body size accounts for some of the division of labour in bumblebee colonies (Jandt *et al.* 2009), there is great inter-individual variability. Although larger individuals bring in more forage on average, this is only because there is an increase in maximum returns. In *B. terrestris*, Goulson *et al.* (2002) found that small individuals (4mm thorax width) transport 10-50mg of pollen per trip, whereas large individuals (5.5mm thorax width) transport 10-150mg. The most obvious explanation is that successfully finding forage does not

depend on size. However, if one assumes individuals only return fully laden, then some large individuals must be poorly equipped to handle large loads.

I suggest that if this is the case, then it might be explained by the diversity in relative size proportions within a colony, which leads to more or less efficient fliers. I would predict that if two bees of similar size are compared, the forager would be the more efficient flier, for instance by having relatively larger wings. This decreases the cost of loads, or allows greater loads to be carried, because the bees are not as close to their maximum lift potential (Roberts *et al.* 2004). On top of division of labour based on body size, there is stochasticity in individuals' task selections, and individuals are more likely to do one day what they were doing the previous day (Jandt *et al.* 2009). Data graciously provided to me by Levente Orbán (Dept. Psychology) on the frequency of individuals' foraging trips indicated that most foraging trips are performed by a very few individuals. This result also suggested that foraging individuals were more likely to forage again, suggesting positive feedback. This corroborates the result of Jandt *et al.*, and additionally suggests that the positive feedback may be related to individual rewards. A poor forager should not find foraging as rewarding (bees get excited by good forage, Mapald *et al.* 2008), and so should not repeat the same task. Future work can examine whether foragers receiving poor rewards are more likely to switch to another task, and whether this is influenced by individual morphological differences.

Separately, large individuals might be better foragers because larger bees warm faster, fly hotter, and cool less than smaller bees (Stone 1993). Presumably, this helps make them more efficient foragers, but there may also be significant costs to the more than 5°C range in body temperatures between workers of a similar mass. Variation in T_{head} might

affect neural functioning and enzyme flux rates, both of which can influence foraging efficiency. Because enzyme activities are assayed at the same temperature, little attention has been paid to the specific effect of variation in body temperature on enzyme activities. In Chapter 4, neither the correlations of enzyme activity to MR, nor the calculated flux rates, took into account differences in body temperature, which may alter the estimate of the enzyme V_{\max} in the flying individual.

HK V_{\max} from the flight muscle of the bug *Dipetelogaster maximus* increases at least until 37°C (Scaraffia & Gerez de Burgos 2000). At higher temperatures, its inhibition by ATP is reduced, but its K_m for glucose increases. Unfortunately, data on the thermal profile of HK is scarce, and Scaraffia and Gerez de Burgos only tested three temperatures; the relative enzyme activity is plotted in Fig. 5.3. In order to illustrate my point, I adapt their data with some assumptions. First, I assume that 37°C is the optimal enzyme temperature (T_{optimum}) in *Dip. maximus*, even though Scaraffia and Gerez de Burgos (2000) did not test this. I also assume that the thermal profile is approximately Gaussian (normal distribution), such that activity is lost at high temperatures at the same rate as low temperatures. This is a reasonable approximation provided that enzyme activity is temperature-dependent and exhibits a temperature optimum. The temperature-specific HK activity (T_{HK}) would then be described as,

$$T_{\text{HK}} = 0.22e^{\frac{-(T_{\text{thorax}} - T_{\text{optimum}})^2}{2k^2}} \quad \text{Eq. 1}$$

where k is a dimensionless scaling constant that sets the width of the peak ($k=5.5$). The form of the profile is probably conservative, since activity usually degrades faster above T_{optimum} (Peterson *et al.* 2004). In order to make comparisons in bumblebees, I assume that bumblebee T_{HK} is equivalent to *Dip. maximus*, except that the T_{optimum} is 1°C higher, for

reasons of internal consistency (explained below). When the derived bumblebee T_{HK} is coplotted with the distribution of thorax temperatures (Chapter 3), the distributions match quite well (Fig. 5.3). Individuals within one standard deviation (sd) of the peak at 38°C operate within at least 95% of the HK V_{max} measured at 37°C, and within two sd, activity is at least 80%. The very few individuals at the extreme temperatures are likely to be the ones who are seriously challenged, as they may have as little as 65% of available HK capacity. The ‘*in vivo*’ activity of HK calculated by the V_{max} at 37°C, or dynamically according to T_{HK} (Eq. 1), differed by 10-15% , but in a few extreme cases, as much as 30%. These latter bees ostensibly operated at >90% V_{max} (using $T_{optimum}=37°C$, these bees fly at 120% capacity, which is why T_{HK} was shifted). Interestingly, Heinrich (1979) notes that bumblebees are unable to fly if their muscle temperature is reduced to 30°C, which in this case would suggest 50% enzyme capacity. Coincidentally, 50% enzyme capacity was the threshold for flight I found in maturing adults (Chapter 2). The initiation of flight may be dependent on metabolic rates high enough to generate sustained high muscle temperature. In terms of the colony, this suggests that when taking into account T_{HK} , some individuals may have narrower operating ranges than expected and be more challenged in flight, especially the largest individuals and the least efficient fliers.

In conclusion, this study reveals a small part of the complex regulation of insect flight performance and metabolic supply. I do not find training differences in enzyme capacity, which may mean that adults are constrained by their development. However, physiological regulation by behaviour may accomplish some of the same goals, and plasticity in response to temperature should be examined. The diversity of flight phenotypes resulting from idiosyncrasies in body plans corroborates strong selection on consistent

scaling (Frankino *et al.* 2005; Houle *et al.* 2003; Darveau *et al.* 2005a). Nonetheless, there is great diversity in the regulation of size in bumblebee workers between colonies (Couvillon *et al.* 2010). Individuality among workers may help to explain their task selection and colony performance, especially in changing environments. Borrowing analytic methods from other fields, as we have borrowed their laboratory techniques, physiologists are the best poised to understand the consequences of individuality. Variation and plasticity, long the basic unit of genetics, should now also inform physiology.

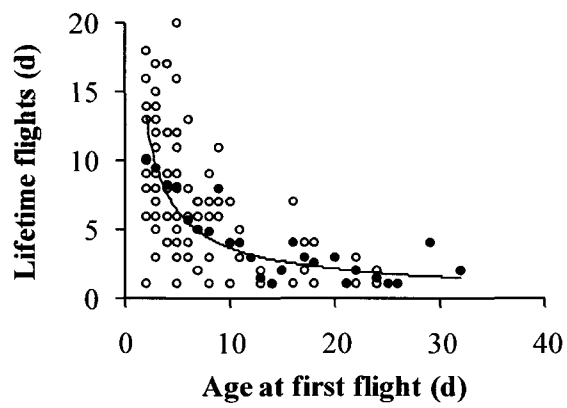


Figure 5.1 Relationship of lifetime flights to age at first flight. Individual (white) and mean first flight age-specific data (black) are compared. Individuals with many lifetime flights started flying at a younger age.

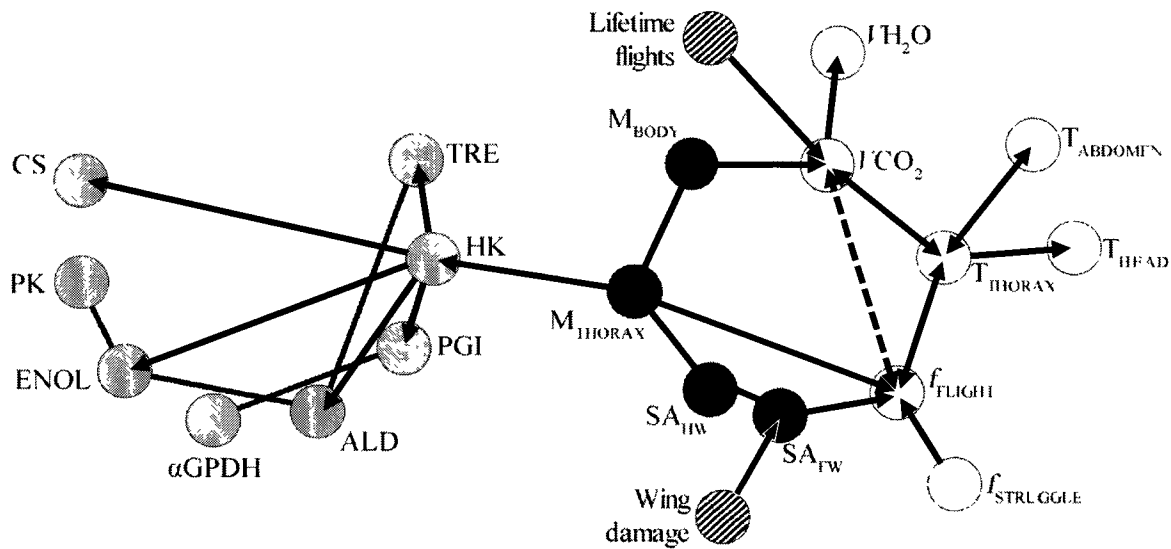


Figure 5.2 Hypothesised directional paths among flight variables in this study. Graph is reproduced from Chapter 4, and coloured corresponding to body size (black), enzyme capacity (grey), and flight parameters (white). Striped nodes are experimental manipulations. Arrows indicate the direction of hypothesised causation. The dashed arrow indicates a correlation that is only observed mass-independently.

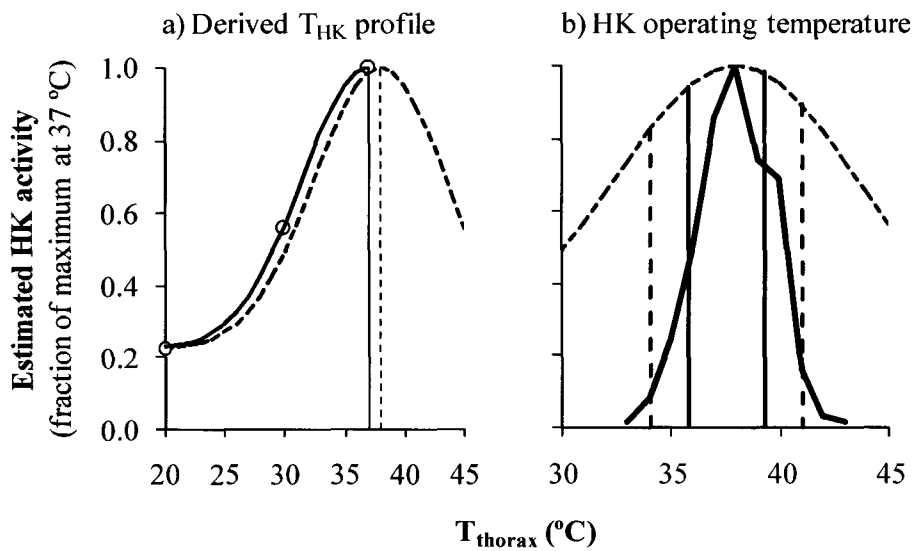


Figure 5.3 Variation in temperature-dependent hexokinase (HK) activity over the range of thorax temperatures (T_{thorax}) found within the population. In panel a, data (open circles) obtained from Scaraffia and Gerez de Burgos (2000) is fit to a Gaussian distribution approximation of the thermal profile (T_{HK} , solid line), with an optimum temperature of 37°C. The same profile would suggest bees operate at fractional velocities >1 , and so the peak was shifted to 38°C (dashed line). In panel b, the profile is coplotted with the distribution of T_{thorax} (bold black line) found in bumblebee workers. Solid and dashed vertical lines represent mean \pm one or two standard deviations of the distribution of T_{thorax} . For most workers, T_{HK} is a high percentage of the V_{max} measured at optimal conditions, but thermal effects on HK may account for up to 20% lower activities.

LITERATURE CITED

- Addo-Bediako A, Chown SL, Gaston KJ. 2000. Thermal tolerance, climatic variability and latitude. *Proc. R. Soc. Lond. B* 267:739-45.
- Almbro M, Kullberg C. Impaired escape flight ability in butterflies due to low flight muscle ratio prior to hibernation. *J. Exp. Biol.* 211:24-8.
- Anderson M, Finlayson LH. 1976. The effect of exercise on the growth of mitochondria and myofibrils in the flight muscles of the tsetse fly, *Glossina morsitans*. *J. Morph.* 150:321-6.
- Balboni ER. 1967. The respiratory metabolism of insect flight muscle during adult maturation. *J. Insect Physiol.* 13:1849-56.
- Bartelink AKM, Gijzel WP, de Kort CAD, Kroon AM. 1975. Mitochondrial formation in the developing flight muscles of the colorado beetle. *Dev. Biol.* 42:262-73.
- Bartholomew GA, Casey TM. 1978. Oxygen consumption of moths during rest, pre-flight warm-up, and flight in relation to body size and wing morphology. *J. Exp. Biol.* 76:11-25.
- Beenackers AMT, van den Broek ATM, de Ronde TJA. 1974. Development of catabolic pathways in insect flight muscles. A comparative study. *J. Insect Physiol.* 21:849-59.
- Bélanger K. 2009. Variation interindividuelle du taux métabolique chez *Bombus impatiens*. Répétabilité et relation avec la fréquence de battement des ailes et la morphologie des ailes. B.Sc. Thesis, University of Ottawa.
- Buchwald R, Dudley R. 2010. Limits to vertical force and power production in bumblebees (Hymenoptera: *Bombus impatiens*). *J. Exp. Biol.* 213:426-32.
- Burness GP, Leary SC, Hochachka PW, Moyes CS. 1999. Allometric scaling of RNA, DNA and enzyme levels: An intra-specific study. *Am. J. Physiol.* R277:1164-70.
- Campbell & Birt 1972. Studies on the appearance of soluble α -glycerophosphate dehydrogenase activity during the development of the sheep blowfly *Lucilia*. *Insect Biochem.* 2:279-96.
- Candy DJ, Becker A, Wegener G. 1997. Coordination and integration of metabolism in insect flight. *Comp. Biochem. Physiol.* B117:497-512.
- Cartar RV. 1992. Morphological senescence and longevity: An experiment relating wing wear and life span in foraging wild bumble bees. *J. Anim. Ecol.* 61:225-31.
- Casey TM. 1976. Flight energetics of sphinx moths: power input during hovering flight. *J. Exp. Biol.* 64:529-43.
- Chen X, Haunerland NH. 1993. Fatty acid binding protein expression in locust flight muscle. Induction by flight, adipokinetic hormone, and low density lipophorin. *Insect Biochem. Molec Biol.* 24:573-9.
- Chesky JA. 1974. The intrinsic nature of the loss of actomyosin ATPase activity during senescence in the male house fly, *Musca domestica* L. *Mech. Ageing Dev.* 3:241-4.
- Clark AG, Keith LE. 1989. Rapid enzyme kinetic assays of individuals *Drosophila* and comparisons of field-caught *D. melanogaster* and *D. simulans*. *Biochem. Genetics* 27:263-77.
- Clark AG, Wang L. 1994. Comparative evolutionary analysis of metabolism in nine *Drosophila* species. *Evolution* 48:1230-43.
- Coelho JR. 1991. The effect of thorax temperature on force production during tethered flight in honeybee (*Apis mellifera*) drones, workers, and queens. *Physiol. Zool.* 64:823-35.

- Collatz K-G, Stammer G, Wilps H, Mehler L. 1981. Programmed loss of flight ability in the early adult life of the blowfly *Phormia terraenovae* as a possible mechanism of intra-specific niche building with respect to the duration of life. *Comp. Biochem. Physiol.* 68A:571-7.
- Combes SA, Crall JD, Mukherjee S. Dynamics of animal movement in an ecological context: Dragonfly wing damage reduces flight performance and predation success. *Biol. Lett.* 6:426-9.
- Couvillon MJ, Jandt JM, Duong N, Dornhaus A. 2010. Ontogeny of worker body size distribution in bumble bee (*Bombus impatiens*) colonies. *Ecol. Entomol.* 35:424-35.
- Darveau CA, Hochachka PW, Welch KC, Roubik DW, Suarez RK. 2005a. Allometric scaling of flight energetics in Panamanian orchid bees: a comparative phylogenetic approach. *J. Exp. Biol.* 208:3581-91.
- Darveau CA, Hochachka PW, Roubik DW, Suarez RK. 2005b. Allometric scaling of flight energetics in orchid bees: Evolution of flux capacities and flux rates. *J. Exp. Biol.* 208:3592-602.
- Darveau CA, Suarez RK, Andrews RD, Hochachka PW. 2002. Allometric cascades - Reply. *Nature* 421:714.
- Davies R, Moyes CD. 2007. Allometric scaling in centrarchid fish: Origins of intra- and inter-specific variation in oxidative and glycolytic enzyme levels in muscle. *J. Exp. Biol.* 210:3798-804.
- Eanes WF, Merritt TJS, Flowers JM, Kumagai S, Sezgin E, Zhu CT. 2006. Flux control and excess capacity in the enzymes of glycolysis and their relationship to flight metabolism in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 103:19413-8.
- Ellington CP, Machin KE, Casey TM. 1990. Oxygen consumption of bumblebees in forward flight. *Nature* 347:472-3.
- Ellington CP. 1984. The aerodynamics of hovering insect flight. II. Morphological parameters. *Phil. Trans. R. Soc. London* 305:17-40.
- Fernandes J, Bate M, VijayRaghavan K. 1991. Development of the indirect flight muscles of *Drosophila*. *Development* 113:67-77.
- Feuerbacher E, Fewell JH, Roberts SP, Smith EF, Harrison JF. 2003. Effects of load type (pollen or nectar) and load mass on hovering metabolic rate and mechanical power output in the honey bee *Apis mellifera*. *J. Exp. Biol.* 206:1855-65.
- Fielding K, Hepburn HR, Chandler HD. 1980. On the development of flight competence in worker honeybees. *Comp. Biochem. Physiol.* 65A:129-33.
- Fischer H, Kutsch W. 2000. Relationships between body mass, motor output and flight variables during free flight of juvenile and mature adult locusts, *Schistocerca gregaria*. *J. Exp. Biol.* 203:2723-35.
- Frankino WA, Zwaan BJ, Stern DL, Brakefield PM. 2005. Natural selection and developmental constraints in the evolution of allometries. *Science* 307:718-20.
- Frazier MR, Harrison JF, Kirkton SD, Roberts SP. 2008. Cold rearing improves cold-flight performance in *Drosophila* via changes in wing morphology. *J. Exp. Biol.* 211:2116-22.
- Fuente A de la, Bing N, Hoeschele I, Mendes P. 2004. Discovery of meaningful associations in genomic data using partial correlation coefficients. *Bioinformatics* 20:3565-74.
- Garland T, Adolph SC. 1994. Why not to do two-species comparative studies: Limitations on inferring adaptation. *Physiol. Zool.* 67:797-828.

- Garland T. 1984. Physiological correlates of locomotory performance in a lizard: An allometric approach. *Am. J. Physiol.* R247:806-15.
- Garland TG, Carter PA. 1994. Evolutionary physiology. *Annu. Rev. Physiol.* 56:579-621.
- Giannini KM. 1997. Labor division in *Melipona compressipes fasciculata* Smith (Hymenoptera: Apidae: Meliponinae). *An. Soc. Entomol. Brasil* 26:153-62.
- Gilmour KM, Ellington CP. 1993. Power output of glycerinated bumblebee flight muscle. *J. Exp. Biol.* 183:77-100.
- Goulson D, Peat J, Stout JC, Tucker J, Darvill B, Derwent LC, Hughes WO. 2002. Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? *Anim. Behav.* 64:123-30.
- Graham JWA, Williams TCR, Morgan M, Fernie AR, Ratcliffe RG, Sweetlove LJ. 2007. Glycolytic enzymes associate dynamically with mitochondria in response to respiratory demand and support substrate channeling. *The Plant Cell* 19:3723-38.
- Gray JR, Robertson RM. 1994. Activity of the forewing stretch receptor in immature and mature adult locusts. *J. Comp. Physiol.* 175A:425.
- Gunn A, Gatehouse AG. 1988. The development of enzymes involved in flight muscle metabolism in *Spodoptera exempta* and *Mythimna separata*. *Comp. Biochem. Physiol.* 91B:315-24.
- Haas CA, Cartar RV. 2008. Robust flight performance of bumble bees with artificially induced wing wear. *Can. J. Zool.* 86:668-75.
- Hanauer-Thieser U, Nachtigall W. 1995. Flight of the honey bee VI: Energetics of wind tunnel exhaustion flight at defined fuel content, speed adaptation and aerodynamics. *J. Comp. Physiol. B* 165:471-83.
- Harrison JF, Nielsen DI, Page RE. 1996a. Malate dehydrogenase phenotype, temperature and colony effects on flight metabolic rate in the honey-bee, *Apis mellifera*. *Funct. Ecol.* 10:81-88.
- Harrison JF, Fewell JH, Roberts SP, Hall HG. 1996b. Achievement of thermal stability by varying metabolic heat production in flying honeybees. *Science* 274:88-90.
- Harrison JF and Fewell JH. 2002. Environmental and genetic influences on flight metabolic rate in the honey bee, *Apis mellifera*. *Comp. Biochem. Physiol.* 133A:323-33.
- Harrison JM. 1986. Caste-specific changes in honeybee flight capacity. *Physiol. Zool.* 59:175-87.
- Hauerland NH. 1997. Transport and utilization of lipids in insect flight muscles. *Comp. Biochem. Physiol.* 117B:475-82.
- Hedenström A, Ellington CP, Wolf TJ. 2001. Wing wear, aerodynamics and flight energetics in bumblebees (*bombus terrestris*): an experimental study. *Funct. Ecol.* 15:417-22.
- Heinrich B. 1976. Heat exchange in relation to blood flow between thorax and abdomen in bumblebees. *J. Exp. Biol.* 64:561-85.
- Heinrich B. 1979. Bumblebee Economics. Cambridge: Harvard University Press. 245 p.
- Herold RC, Borei H. 1963. Cytochrome changes during honey bee flight muscle development. *Develop. Biol.* 8:67-79.
- Herold RC. 1965. Development and ultrastructural changes of sarcosomes during honey bee flight muscle development. *Develop. Biol.* 12:269-86.
- Hesselberg T, Lehmann FO. 2009. The role of experience in flight behaviour of *Drosophila*. *J. Exp. Biol.* 212:3377-86.

- Higginson AD, Barnard CJ. 2004. Accumulating wing damage affects foraging decisions in honeybees (*Apis mellifera* L.). *Ecol. Entomol.* 29:52-9.
- Holmes RP, Birt LM. 1977. Synthesis of nucleic acids during the metamorphosis of the blowfly *Lucilia cuprina*. *Insect Biochem.* 7:491-501.
- Houle D, Mezey J, Galpern P, Carter A. 2003. Automated measurement of *Drosophila* wings. *BMC Evol. Biol.* 3:25.
- Huey RB, Bennet AF. 2008. Bart's familiar quotations: The enduring biological wisdom of George A. Bartholomew. *Physiol. Biochem. Zool.* 81:519-25. (Source of Bartholomew quotes in text)
- Hunter AS, Cediell N. 1970. Effects of temperature on *Drosophila* VI. Respiratory enzymes. *Comp. Biochem. Physiol.* 37:243-9.
- Iwamoto H, Inoue K, Yagi N. 2006. Evolution of long-range myofibrillar crystallinity in insect muscle as examined by X-ray cryomicrodiffraction. *Proc. R. Soc. B* 273:677-85.
- Jandt JM, Huang E, Dornhaus A. 2009. Weak specialization of workers inside a bumble bee (*Bombus impatiens*) nest. *Behav. Ecol. Sociobiol.* 63:1829-36.
- Jantzen B, Eisner T. 2008. Hindwings are unnecessary for flight but essential for execution of normal evasive flight in Lepidoptera. *Proc. Natl. Acad. Sci. USA* 105:16636-40.
- Joanisse, D.R. and Storey, K.B. 1994. Enzyme activity profiles in an overwintering population of freeze avoiding gall moth larvae *Epiblema scudderiana*. *Can. J. Zool.* 72:1079-86.
- Johnston IA, Moon TW. 1980. Exercise training in skeletal muscle of brook trout (*Salvelinus fontinalis*). *J. Exp. Biol.* 87:177-94.
- Joos B, Young PA, Casey TM. 1991. Wingstroke frequency of foraging and hovering bumblebees in relation to morphology and temperature. *Physiol. Entomol.* 16:191-200.
- Koster Asj, Beenackers AMTh. 1979. Effect of denervation on enzyme levels in flight muscles of the adult migratory locust. *J. Insect Physiol.* 25:389-92.
- Lachenicht MW, Clusella-Trullas S, Boardman L, Le Roux C, Terblance JS. 2010. Effects of acclimation temperature on thermal tolerance, locomotion performance and respiratory metabolism in *Acheta domesticus* L. (Orthoptera: Gryllidae). *J. Insect Physiol.* 56: 822-30.
- Laurie-Ahlberg CC, Barnes PT, Curtsinger JW, Emigh TH, Karlin B, Morris R, Norman RA, Wilton AN. 1985. Genetic variability of flight metabolism in *Drosophila melanogaster*. II. Relationship between power output and enzyme activity levels. *Genetics* 111:845-68.
- Lehmann FO, Dickinson MH, Staunton J. 2000. The scaling of carbon dioxide release and respiratory water loss in flying fruit flies (*Drosophila* spp.). *J. Exp. Biol.* 1613-1624.
- Lehmann FO, Dickinson MH. 1997. The changes in power requirements and muscle efficiency during elevated force production in the fruit fly *Drosophila melanogaster*. *J. Exp. Biol.* 200:1133-43.
- Lehmann FO. 2001. Matching spiracle opening to metabolic need during flight in *Drosophila*. *Science* 294, 1926-1929.
- Licea S. 2010. B.Sc. Thesis, University of Ottawa.
- Magwera T, Pamplona R, Miwa S, Martinez-Diaz P, Portero-Otin M, Brand MD, Partridge L. 2006. Flight activity, mortality rates, and lipoxidative damage in *Drosophila*. *J. Gerontol.* 61A:136-45.

- Mangum CP, Hochachka PW. 1998. New direction in comparative physiology and biochemistry: Mechanisms, adaptations, and evolution. *Physiol. Zool.* 71:471-84.
- Mapalad KS, Leu D, Nieh JC. 2008. Bumble bees heat up for high quality pollen. *J. Exp. Biol.* 211:2239-42.
- Marden JH. 1989. Bodybuilding dragonflies: costs and benefits of maximizing flight muscle. *Physiol. Zool.* 62:505-21.
- Marden JH. 2000. Variability in the size, composition, and function of insect flight muscles. *Ann. Rev. Physiol.* 62:157-78.
- Moffatt L. 2001. Changes in the metabolic rate of the foraging honeybee: effect of the carried weight or of the reward rate? *J. Comp. Physiol.* 186A:299-306.
- Montooth KL, Marden JH, Clark AG. 2003. Mapping determinants of variation in energy metabolism, respiration and flight in *Drosophila*. *Genetics* 165:623-35.
- Mykles DL, Ghalambor CK, Stillman JH, Tomanek L. 2010. Grand challenges in comparative physiology: Integration across disciplines and across levels of biological organization. *Integ. Comp. Biol.* 50:6-16.
- Newsholme EA, Crabtree B, Higgins SJ, Thornton SD, Start C. 1972. The activities of fructose diphosphatase in flight muscles from the bumble-bee and the role of this enzyme in heat generation. *Biochem. J.* 128:89-97.
- Niitepõld K, Smith AD, Osborne JL, Reynolds DR, Carreck NL, Martin AP, Marden JH, Ovaskainen O, Hanski I. 2009. Flight metabolic rate and Pgi genotype influence butterfly dispersal rate in the field. *Ecology* 90:2223-32.
- Niitepõld K. 2010. Genotype by temperature interactions in the metabolic rate of the Glanville fritillary butterfly. *J. Exp. Biol.* 213:1042-8.
- Niven JE, Scharlemann JPW. 2005. Do insect metabolic rates at rest and during flight scale with body mass? *Biol. Lett.* 1:346-9.
- Payne CM, Lavery TM, Lachance MA. 2003. The frequency of multiple paternity in bumble bee (*Bombus*) colonies based on microsatellite DNA at the B10 locus. *Insect. Soc.* 50:375-8.
- Petersen B, Lundgren L, Wilson L. 1956-1957. The development of flight capacity in a butterfly. *Behaviour* 10:324-39.
- Peterson ME, Eisenthal R, Danson MJ, Spence A, Daniel RM. 2004. A new intrinsic thermal parameter for enzymes reveals true temperature optima. *J. Biol. Chem.* 279:20717-22.
- Petratis PS, Dunham AE, Niewiarowski PH. 1996. Inferring multiple causality: the limitations of path analysis. *Funct. Ecol.* 10:421-31.
- Pierce VA, Crawford DL. 1994. Phylogenetic analysis of glycolytic enzyme expression. *Science* 276:256-9.
- Pyke GH. 1978. Optimal body size in bumblebees. *Oecologia* 34:255-66.
- R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. ISBN 3-900051-07-0, URL: <http://www.R-project.org>.
- Rasband, W.S. 1997-2009. ImageJ. U. S. National Institutes of Health, Bethesda, Maryland, USA. <http://rsb.info.nih.gov/ij/>.
- Rezende EL, Gomes FR, Chappell MA, Garland T. 2009. Running behavior and its energy cost in mice selectively bred for high voluntary locomotor activity. *Physiol. Biochem. Zool.* 82:662-79.

- Ring RA. 1973. Changes in dry weight, protein, and nucleic acid content during diapause and normal development of the blowfly, *Lucilia sericata*. *J. Insect Physiol.* 19:481-94.
- Roberts SP, Harrison JF, Dudley R. 2004. Allometry of kinematics and energetics in carpenter bees (*Xylocopa varipuncta*) hovering in variable-density gases. *J. Exp. Biol.* 207:993-1004.
- Roberts SP, Harrison JF. 1999. Mechanisms of thermal stability during flight in the honeybee *Apis mellifera*. *J. Exp. Biol.* 202:1523-33.
- Roy C. 2010. Maturation of flight muscle energetics: mitochondrial respiratory capacities in *Bombus impatiens*. BSc. Thesis. University of Ottawa.
- Scaraffia PY, Gerez de Burgos NM. 2000. Effects of temperature and pH on hexokinase from the flight muscles of *Dipetalogaster maximus* (Hemiptera: Reduviidae). *J. Med. Entomol.* 37:689-94.
- Shingleton AW, Estep CM, Driscoll MV, Dworkin I. 2009. Many ways to be small: Different environmental regulators of size generate distinct scaling relationships in *Drosophila melanogaster*. *Proc. R. Soc. B* 276:2625-33.
- Schippers MP, Dukas R, McClelland GB. 2010. Lifetime- and caste-specific changes in flight metabolic rate and muscle biochemistry of honeybees, *Apis mellifera*. *J. Comp. Physiol.* 180B:45-55.
- Schippers MP, Dukas R, Smith RW, Wang J, Smolen K, McClelland GB. 2006. Lifetime performance in foraging honeybees: behaviour and physiology. *J. Exp. Biol.* 209:3828-36.
- Shipley B. 2002. Cause and correlation in biology: A user's guide to path analysis, structural equations and causal inference. Cambridge University Press. 332 pgs.
- Sirover MA. 1999. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim. Biophys. Acta* 1432:159-84.
- Skandalis DA, Tattersall GJ, Prager S, Richards MH. 2009. Body size and shape of the large carpenter bee, *Xylocopa virginica* (L.) (Hymenoptera: Apidae). *J. Kansas Entomol. Soc.* 82:30-42.
- Socha R, Sula J. 2008. Regulation of the development of flight muscles in long-winged adults of the flightless bug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). *Eur. J. Entomol.* 105:575-83.s
- Srygley RB, Kingsolver JG. 2000. Effects of weight loading on flight performance and survival of palatable Neotropical *Anartia fatima* butterflies. *Biol. J. Linnean Soc.* 70:707-25.
- Staples JF, Suarez RK. 1997. Honeybee flight muscle phosphoglucose isomerase: matching enzyme capacities to flux requirements at a near-equilibrium reaction. *J. Exp. Biol.* 200:1247-54.
- Stone GN. 1993. Endothermy in the solitary bee *Anthophora plumipes*: independent measures of thermoregulatory ability, costs of warm-up and the role of body size. *J. Exp. Biol.* 174:299-320.
- Suarez RK. 1996. Upper limits to mass-specific metabolic rates. *Annu. Rev. Physiol.* 58:583-605.
- Suarez RK. 1998. Oxygen and the upper limits to animal design and performance. *J. Exp. Biol.* 201:1065-1072.
- Suarez RK. 2000. Energy metabolism during insect flight: biochemical design and physiological performance. *Physiol. Biochem. Zool.* 73:765-71.

- Suarez RK, Darveau CA, Welch KC, O'Brien DM, Roubik DW, Hochachka PW. 2005. Energy metabolism in orchid bee flight muscles: Carbohydrate fuels all. *J. Exp. Biol.* 208:3573-9.
- Suarez RK, Lighton JRB, Joos B, Roberts SP, Harrison JF. 1996. Energy metabolism, enzymatic flux capacities, and metabolic flux rates in flying honeybees. *Proc. Natl. Acad. Sci. USA* 93:12616-20.
- Sullivan DT, MacIntyre R, Fuda N, Fiori J, Barilla J, Ramizel L. 2003. Analysis of glycolytic enzyme co-localization in *Drosophila* flight muscle. *J. Exp. Biol.* 206:2031-8.
- Trotta V, Pertoldi C, Rudoy A, Manenti T, Cavicchi S, Guerra D. 2010. Thermal plasticity of wing size and shape in *Drosophila melanogaster*, *D. simulans* and their hybrids. *Clim. Res.* 43:71-9.
- van Marrewijk WJA, Schrikker AEM, Beenackers AMT. 1980. Contents of nucleic and amino acids and rate of protein synthesis in developing flight muscles of *Locusta migratoria*. *Comp. Biochem. Physiol.* 65B:251-7.
- Vance JT, Williams JB, Elekonich MM, Roberts SP. 2009. The effects of age and behavioral development on honey bee (*Apis mellifera*) flight performance. *J. Exp. Biol.* 212:2804-11.
- Wojtas K, Slepecky N, von Kalm L, Sullivan D. 1997. Flight muscle function in *Drosophila* requires colocalization of glycolytic enzymes. *Mol. Biol. Cell.* 8:1665-75.
- Wolschin F, Amdam GV. 2007. Plasticity and robustness of protein patterns during reversible development in the honey bee (*Apis mellifera*). *Anal. Bioanal. Chem.* 389:1095-100.
- Woods WA, Heinrich B, Stevenson RD. 2005. Honeybee flight metabolic rate: does it depend upon air temperature? *J. Exp. Biol.* 208:1161-73.
- Yang TH, Somero GN. 1996. Activity of lactate dehydrogenase but not its concentration of messenger RNA increases with body size in barred sand bass, *Paralabrax nebulifer* (Teleostei). *Biol. Bull.* 191:155-8.
- Zar JH. 1999. Biostatistical Analysis, 4th ed. Prentice Hall, New Jersey.